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1. Document ID: US 20050054106 A1

L29: Entry 1 of 16 File: PGPB Mar 10, 2005

DOCUMENT-IDENTIFIER: US 20050054106 A1
TITLE: DNA recombination in eukaryotic cells by the bacteriophage PHIC31 recombination system

Detail Description Paragraph:
[0025] The term "**isolated**", when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. In particular, an **isolated** gene is separated from open reading frames which flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

Detail Description Paragraph:
[0026] The term "naturally-occurring" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be **isolated** from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

Detail Description Paragraph:
[0031] "Promoter" refers to a region of DNA involved in binding the RNA polymerase to initiate transcription. An "**inducible promoter**" refers to a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, **temperature**, pH, transcription factors and chemicals.

Detail Description Paragraph:
[0057] A promoter can be derived from a gene that is under investigation, or can be a heterologous promoter that is obtained from a different gene, or from a different species. Where direct expression of a gene in all **tissues** of a transgenic plant or other organism is desired, one can use a "constitutive" promoter, which is generally active under most environmental conditions and states of development or

cell differentiation. Suitable constitutive promoters for use in plants include, for example, the cauliflower mosaic virus (CaMV) 35S transcription initiation region and region VI promoters, the 1'- or 2'-promoter derived from T-DNA of Agrobacterium tumefaciens, and other promoters active in plant cells that are known to those of skill in the art. Other suitable promoters include the full-length transcript promoter from Figwort mosaic virus, actin promoters, histone promoters, tubulin promoters, or the mannopine synthase promoter (MAS). Other constitutive plant promoters include various ubiquitin or polyubiquitin promoters derived from, inter alia, Arabidopsis (Sun and Callis, Plant J., 11(5):1017-1027 (1997)), the mas, Mac or DoubleMac promoters (described in U.S. Pat. No. 5,106,739 and by Comai et al., Plant Mol. Biol. 15:373-381 (1990)) and other transcription initiation regions from various plant genes known to those of skill in the art. Such genes include for example, ACT11 from Arabidopsis (Huang et al., Plant Mol. Biol. 33:125-139 (1996)), Cat3 from Arabidopsis (GenBank No. U43147, Zhong et al., Mol. Gen. Genet. 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from Brassica napus (Genbank No. X74782, Solocombe et al., Plant Physiol. 104:1167-1176 (1994)), GPc1 from maize (GenBank No. X15596, Martinez et al., J. Mol. Biol 208:551-565 (1989)), and Gpc2 from maize (GenBank No. U45855, Manjunath et al., Plant Mol. Biol. 33:97-112 (1997)). Useful promoters for plants also include those obtained from Ti- or Ri-plasmids, from plant cells, plant viruses or other hosts where the promoters are found to be functional in plants. Bacterial promoters that function in plants, and thus are suitable for use in the methods of the invention include the octopine synthetase promoter, the nopaline synthase promoter, and the manopine synthetase promoter. Suitable endogenous plant promoters include the ribulose-1,6-biphosphate (RUBP) carboxylase small subunit (ssu) promoter, the .alpha.-conglycinin promoter, the phaseolin promoter, the ADH promoter, and heat-shock promoters.

Detail Description Paragraph:

[0059] Alternatively, one can use a promoter that directs expression of a gene of interest in a specific **tissue** or is otherwise under more precise environmental or developmental control. Such **promoters are referred to here as "inducible" or "repressible" promoters**. Examples of environmental conditions that may effect transcription by **inducible promoters** include pathogen attack, anaerobic conditions, ethylene or the presence of light. Promoters under developmental control include promoters that initiate transcription only in certain **tissues**, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an **inducible promoter** may become fully or partially constitutive in certain locations. **Inducible promoters** are often used to control expression of the recombinase gene, thus allowing one to control the timing of the recombination reaction. Examples of **tissue**-specific plant promoters under developmental control include promoters that initiate transcription only in certain **tissues**, such as fruit, seeds, or flowers. The **tissue**-specific E8 promoter from tomato is particularly useful for directing gene expression so that a desired gene product is located in fruits. See, e.g., Lincoln et al. (1988) Proc. Nat'l. Acad. Sci. USA 84: 2793-2797; Deikman et al. (1988) EMBO J. 7: 3315-3320; Deikman et al. (1992) Plant Physiol. 100: 2013-2017. Other suitable promoters include those from genes encoding embryonic storage proteins. Examples of environmental conditions that may affect transcription by **inducible promoters** include anaerobic conditions, elevated **temperature**, or the presence of light. Additional organ-specific, **tissue**-specific and/or **inducible foreign promoters** are also known (see, e.g., references cited in Kuhlemeier et al (1987) Ann. Rev. Plant Physiol. 38:221), including those 1,5-ribulose bisphosphate carboxylase small subunit genes of Arabidopsis thaliana (the "ssu" **promoter), which are light-inducible** and active only in photosynthetic **tissue**, anther-specific promoters (EP 344029), and seed-specific promoters of, for example, Arabidopsis thaliana (Krebbers et al. (1988) Plant Physiol. 87:859). Exemplary green **tissue**-specific promoters include the maize phosphoenol pyruvate carboxylase (PEPC) promoter, small submit ribulose bis-carboxylase promoters (ssRUBISCO) and the chlorophyll a/b binding protein promoters. The promoter may also be a pith-specific promoter, such

as the promoter **isolated** from a plant TrpA gene as described in International Publication No. WO93/07278.

Detail Description Paragraph:
[0062] The construction of polynucleotide constructs generally requires the use of vectors able to replicate in bacteria. A plethora of kits are commercially available for the purification of plasmids from bacteria. For their proper use, follow the manufacturer's instructions (see, for example, EasyPrepJ, FlexiPrepJ, both from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The **isolated** and purified plasmids can then be further manipulated to produce other plasmids, used to transfect cells or incorporated into Agrobacterium tumefaciens to infect and transform plants. Where Agrobacterium is the means of transformation, shuttle vectors are constructed. Cloning in Streptomyces or Bacillus is also possible.

Detail Description Paragraph:
[0067] The polynucleotide constructs that include recombination sites and/or recombinase-encoding genes can be introduced into the target cells and/or organisms by any of the several means known to those of skill in the art. For instance, the DNA constructs can be introduced into plant cells, either in culture or in the organs of a plant by a variety of conventional techniques. For example, the DNA constructs can be introduced directly to plant cells using biolistic methods, such as DNA particle bombardment, or the DNA construct can be introduced using techniques such as electroporation and microinjection of plant cell protoplasts. Particle-mediated transformation techniques (also known as "biolistics") are described in Klein et al., Nature, 327:70-73 (1987); Vasil, V. et al., Bio/Technol. 11:1553-1558 (1993); and Becker, D. et al., Plant J., 5:299-307 (1994). These methods involve penetration of cells by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface. The biolistic PDS-1000 Gene Gun (Biorad, Hercules, Calif.) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells. The process is applicable to a wide range of **tissues** and cells from organisms, including plants, bacteria, fungi, algae, intact animal **tissues**, **tissue** culture cells, and animal embryos. One can employ electronic pulse delivery, which is essentially a mild electroporation format for live **tissues** in animals and patients. Zhao, Advanced Drug Delivery Reviews 17:257-262 (1995).

Detail Description Paragraph:
[0074] Transformed plant cells, derived by any of the above transformation techniques, can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a **tissue** culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts **Isolation** and Culture, Handbook of Plant Cell Culture, pp. 124-176, Macmillian Publishing Company, New York (1983); and in Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, (1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar et al., J. **Tissue** Cult. Meth., 12:145 (1989); McGranahan et al., Plant Cell Rep., 8:512 (1990)), organs, or parts thereof. Such regeneration techniques are described generally in Klee et al., Ann. Rev. of Plant Phys., 38:467-486 (1987).

Detail Description Paragraph:
[0075] The methods are useful for producing transgenic and chimeric animals of most vertebrate species. Such species include, but are not limited to, nonhuman mammals, including rodents such as **mice** and rats, rabbits, ovines such as sheep and goats, porcines such as pigs, and bovines such as cattle and buffalo. Methods of obtaining transgenic animals are described in, for example, Puhler, A., Ed., Genetic Engineering of Animals, VCH Publ., 1993; Murphy and Carter, Eds., Transgenesis

Techniques: Principles and Protocols (Methods in Molecular Biology, Vol. 18), 1993; and Pinkert, C A., Ed., Transgenic Animal Technology: A Laboratory Handbook, Academic Press, 1994. Transgenic fish having specific genetic modifications can also be made using the claimed methods. See, e.g., Iyengar et al. (1996) Transgenic Res. 5: 147-166 for general methods of making transgenic fish.

Detail Description Paragraph:
[0076] One method of obtaining a transgenic or chimeric animal having specific modifications in its genome is to contact fertilized oocytes with a vector that includes the polynucleotide of interest flanked by recombination sites. For some animals, such as **mice** fertilization is performed in vivo and fertilized ova are surgically removed. In other animals, particularly bovines, it is preferably to remove ova from live or slaughterhouse animals and fertilize the ova in vitro. See DeBoer et al., WO 91/08216. In vitro fertilization permits the modifications to be introduced into substantially synchronous cells. Fertilized oocytes are then cultured in vitro until a pre-implantation embryo is obtained containing about 16-150 cells. The 16-32 cell stage of an embryo is described as a morula. Pre-implantation embryos containing more than 32 cells are termed blastocysts. These embryos show the development of a blastocoel cavity, typically at the 64 cell stage. If desired, the presence of a desired exogenous polynucleotide in the embryo cells can be detected by methods known to those of skill in the art. Methods for culturing fertilized oocytes to the pre-implantation stage are described by Gordon et al. (1984) Methods Enzymol. 101: 414; Hogan et al. Manipulation of the **Mouse** Embryo: A Laboratory Manual, C. S. H. L. N.Y. (1986) (**mouse** embryo); Hammer et al. (1985) Nature 315: 680 (rabbit and porcine embryos); Gandolfi et al. (1987) J. Reprod. Fert. 81: 23-28; Rexroad et al. (1988) J. Anim. Sci. 66: 947-953 (ovine embryos) and Eyestone et al. (1989) J. Reprod. Fert. 85: 715-720; Camous et al. (1984) J. Reprod. Fert. 72: 779-785; and Heyman et al. (1987) Theriogenology 27: 5968 (bovine embryos). Sometimes pre-implantation embryos are stored frozen for a period pending implantation. Pre-implantation embryos are transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is integrated. Chimeric mammals can be bred to form true germline transgenic animals.

Detail Description Paragraph:
[0088] The 84 bp .PHI.C31 attP site (abbreviated as PP'), **isolated** as an ApaI-SacI fragment from pHS282 (Thorpe & Smith (1998) Proc. Nat'l. Acad. Sci. USA 95:5505-5510) was cloned into the same sites of the S. pombe integrating vector pJK148 (Keeney & Boeke (1994) Genetics 136:849-856) to make pLT44. This plasmid was targeted to the S. pombe leu1-32 allele by lithium acetate mediated transformation with NdeI cut DNA. The recipient host FY527 (h.sup.- ade6-M216 his3-D1 leu1-32 ura4-D18), converted to Leu.sup.+ by homologous recombination with pLT44, was examined by Southern analysis. One Leu.sup.+ transformant, designated FY527attP, was found to contain a single copy of pLT44. Another transformant, designated FY527attPx2, harbors a tandem plasmid insertion.

Detail Description Paragraph:
[0090] The S. pombe ura4.sup.+ gene, excised from pTZura4 (S. Forsburg) on a 1.8 kb EcoRI-BamHI fragment, was inserted into pJK148 cut with the same enzymes to create pLT40. The .PHI.C31 attB site (abbreviated as BB'), **isolated** from pHS21 as a 500 bp BamHI-XbaI fragment, was ligated into pLT40 cut with those enzymes, creating pLT42. Most of the leu1 gene was removed from pLT42 by deleting a XhoI fragment to create pLT45. This removed all but 229 bp of leu1 from pLT45 and reduced its transformation efficiency to that of a plasmid without any leu1 homology. pLT50, which has a second attB site in the same orientation immediately on the other side of ura4, was constructed by first subcloning the attB BamHI-SacI fragment from pLT42 into pUC19, excising it with EcoRI and SalII, and subsequently inserting it into pLT45 cut with EcoRI and XhoI. The second attB site in the final construct was sequenced once on each strand and found to be identical to the first attB site.

Detail Description Paragraph:
[0103] Recombination between the pLT45-encoded .PHI.C31 attB element and the chromosomally situated attP sequence would incorporate the circular DNA into the leu1 locus as depicted in FIG. 1B. If this reaction occurs, XbaI-fractionated genomic DNA from the Ura.sup.+ transformants is probed with leu1 DNA, the 3 kb band will remain unchanged, while the 18 kb band will increase to .about.23 kb (FIG. 1C). Randomly selected Ura.sup.+ colonies were examined by hybridization analysis. Of eight **isolates** derived from experiments where .PHI.C31 integrase gene expression was derepressed by the omission of thiamine, seven showed the presence of this .about.23 kb band. This same size band hybridized to the ura4 probe. This contrasts with the lack of ura4 hybridization with the parental strain, as expected from its ura4-D18 deletion allele. One of these seven **isolates** showed additional bands hybridizing to both probes. This candidate appears to have a DNA rearrangement at the leu1 locus in addition to a site-specific recombination event. The leu1 rearrangement was probably catalyzed by the operative S. pombe homologous recombination system. The remaining **isolate** had not experienced a site-specific recombination event and appeared to have gained uracil prototrophy by recombination between pLT45 and pLT43. Of these eight **isolates**, half were selected as both Ura.sup.+ and His.sup.+, but no significant difference was found between this group and the group selected for Ura.sup.+ only.

Detail Description Paragraph:
[0104] From transformation experiments plated in the presence of vitamin B1, an equal number of Ura.sup.+ transformants was examined by DNA hybridization. The thiamine-repressible Pnmt promoter is expected to limit integrase production, and thereby site-specific integration. Two of the eight Ura.sup.+ candidates **isolated** from this low frequency transformation showed a band of 23 kb hybridizing to leu1 and to the ura4 probe. However, since both probes detected an additional band, they do not represent correct integration events, and we grouped them as class b integrants. In the other six **isolates**, the hybridization patterns are difficult to interpret. In some of them, the 3 kb band was not detected by the leu1 probe, as though the locus has experienced some rearrangement. In many of them, the weak hybridization to ura4 suggests that the Ura.sup.+ phenotype may not be due to the stable maintenance of pLT45 in the genome.

Detail Description Paragraph:
[0105] To ascertain the proportion of transformants maintaining the integrase plasmid in the absence of selection, the blots were re-probed with the integrase gene sequence. Those selected as Ura.sup.+ His.sup.+ would be expected to maintain the plasmid, and did so, as the hybridization revealed. Five of the eight **isolates** selected as Ura.sup.+ without regard to the His phenotype also gave bands hybridizing to the integrase probe. To confirm that loss of int would not affect stable integration, another set of randomly chosen Ura.sup.+ cells were grown non-selectively for a number of generations and screened for His.sup.- progeny that have lost pLT43. The analysis of eight representative Ura.sup.+ His.sup.- clones showed that all had a single copy of pLT45 precisely integrated at the chromosome-situated attP site. The DNA of these integrants did not hybridize with the integrase probe. In contrast, the background frequency Ura.sup.+ clones derived by transformation of pLT45 alone gave the parental configuration of hybridizing bands at the leu1 locus and additional faint bands at 5 kb and 7 kb. These observations are consistent with either integration of pLT45 elsewhere in the genome, or maintenance of the plasmid in some cells despite the lack of a S. pombe replication origin.

Detail Description Paragraph:
[0110] Three Ura.sup.- His.sup.+ clones from each of the three cultures that had been transformed by pLT45 were analyzed by Southern blotting. One **isolate** had a DNA pattern consistent with stable integration of pLT45 into FY527attP. Therefore, in this clone, the Ura.sup.- phenotype was caused by a mutation that did not appreciably alter the restriction pattern, rather than by reversal of the site-

specific recombination reaction. The second clone showed a Southern pattern characteristic of FY527attP lacking a pLT45 insertion, the third had a pattern consistent with a mixture of two types of cells, those like FY527attP without a pLT45 insertion, and those like the FY527attP progenitor strain FY527. The latter structure could arise from intrachromosomal homologous recombination between the leu repeats, reversing the insertion of pLT44 (FIG. 1A). If precise excision of the integrated plasmid DNA occurred in the latter two candidates, the attP site would be regenerated; this would be detectable with PCR. The size of the PCR product was that expected for an intact hybrid site, the presence of the hybrid site was confirmed by sequencing the PCR product. These observations are consistent with the idea that deletion of the ura4 gene occurred by some mechanism other than .PHI.C31-mediated excision.

Detail Description Paragraph:
[0115] The CHO cell line 51YT211 was transfected with the attP-containing plasmid pFY1, which included a selectable marker that confers zeocin resistance (FIG. 2). After being single colony purified twice, six zeocin resistant cell lines were **isolated**. Analysis by Southern DNA hybridization confirmed that each of the six cell lines had at least one molecule of pFY1 integrated into the genome.

Detail Description Paragraph:
[0148] If the two constructs were present in the same genome, the expression of int from the pWP24 bearing chromosome would be expected to produce functional .PHI.C31 integrase to catalyze the recombination between attB and attP sites situated on the pWP29-bearing chromosome. The recombination event would be expected to delete the npt gene from the pWP29 construct and fuse 35S to gus. The resulting configuration would be 35S-attR-gus, where attR is a hybrid site formed by the recombination between attP and attB, also designated as PB' (FIG. 4). The deletion of npt brings gus under the transcription of 35S and would be expected to yield plants with GUS enzyme activity. This activity can be detected through histochemical staining of the plant **tissue**.

Detail Description Paragraph:
[0153] The intensity of staining varied depending on the combination of lines used as parental lines. Those with progeny with a greater proportion of the **tissue** staining blue indicate that the recombination event was more efficient. Conversely, those yielding progeny with less uniform staining indicate that the recombination event was less efficient. This variation among the different progeny pools is probably due to effects caused by the position of integration of the transgenes. Of the two integrase lines, 24.4 appears more efficient in promoting site-specific recombination. This is probably due to a higher level of int gene expression. Staining patterns produced by crossing 24.4 to 29.4 and 29.19 are consistent with the experimental design that int promoted site-specific recombination of attB and attP results in the activation of gus gene activity.

CLAIMS:

2. The eukaryotic cell of claim 1, wherein the recombinase is selected from the group consisting of a bacteriophage .PHI.C31 integrase, a coliphage P4 recombinase, a **Listeria** phage recombinase, a bacteriophage R4 Sre recombinase, a CisA recombinase, an XisF recombinase, and a transposon Tn4451 TnpX recombinase.

☐ 2. Document ID: US 20040234548 A1

L29: Entry 2 of 16 File: PGPB Nov 25, 2004

DOCUMENT-IDENTIFIER: US 20040234548 A1
TITLE: Microbial delivery system

Brief Description of Drawings Paragraph:
[0015] FIG. 3. Results of ELISA analysis of Ara h 2-specific IgG antibodies produced in **mice** following injection of E. coli producing Ara h 2. IgG1 is on the left and IgG2a is on the right.

Brief Description of Drawings Paragraph:
[0016] FIG. 4. Results of ELISA analysis of Ara h 3-specific IgG antibodies produced in **mice** following injection of E. coli producing Ara h 3. IgG1 is on the left and IgG2a is on the right.

Detail Description Paragraph:
[0026] "**Inducible promoter**": **The term "inducible promoter"**, as used herein, means a promoter site which is activated directly by the presence or absence of a chemical agent or indirectly by an environmental stimulus such as **temperature** changes. A promoter is the region of DNA at which the enzyme RNA polymerase binds and initiates the process of gene transcription.

Detail Description Paragraph:
[0051] Methods of inducing transcription include but are not limited to induction by the presence or absence of a chemical agent, **induction using a nutrient starvation inducible promoter, induction using a phosphate starvation inducible promoter and induction using a temperature sensitive inducible promoter**. A particularly preferred system for regulating gene expression utilizes tetracycline controllable expression system. Systems which utilize the tetracycline controllable expression system are commercially available (see for example, Clontech, Palo Alto, Calif.).

Detail Description Paragraph:
[0077] Adjuvants that are known to stimulate Th2 responses are preferably avoided. Particularly preferred adjuvants include, for example, preparations (including heat-killed samples, extracts, partially purified **isolates**, or any other preparation of a microorganism or macroorganism component sufficient to display adjuvant activity) of microorganisms such as Listeria monocytogenes or others (e.g., Bacille Calmette-Guerin [BCG], Corynebacterium species, Mycobacterium species, Rhodococcus species, Eubacteria species, Bortadella species, and Nocardia species), and preparations of nucleic acids that include unmethylated CpG motifs (see, for example, U.S. Pat. No. 5,830,877; and published PCT applications WO 96/02555, WO 98/18810, WO 98/16247, and WO 98/40100, each of which is incorporated herein by reference). Other preferred adjuvants reported to induce Th1-type responses and not Th2-type responses include, for example, Aviridine (N,N-dioctadecyl-N'N'-bis (2-hydroxyethyl)propanediami- ne) and CRL 1005. Particularly preferred are ones that induce IL-12 production, including microbial extracts such as fixed Staphylococcus aureua, Streptococcal preparations, Mycobacterium tuberculosis, lipopolysaccharide (LPS), monophosphoryl lipid A (MPLA) from gram negative bacterial lipopolysaccharides (Richards et al. Infect Immun 1998 June;66 (6):2859-65), listeria monocytogenes, toxoplasma gondii, leishmania major. Some polymers are also adjuvants. For example, polyphosphazenes are described in U.S. Pat. No. 5,500,161 to Andriavnov, et al. These can be used not only to encapsulate the microorganisms but also to enhance the immune response to the antigen.

Detail Description Paragraph:
[0078] If adjuvants are not synthesized by microorganisms in accordance with the present invention, adjuvants which are cytokines may be provided as impure preparations (e.g., **isolates** of cells expressing a cytokine gene, either endogenous or exogenous to the cell), but are preferably provided in purified form. Purified preparations are preferably at least about 90% pure, more preferably at least about 95% pure, and most preferably at least about 99% pure. Alternatively, genes encoding the cytokines or immunological inducing agents may be provided, so that gene expression results in cytokine or immunological inducing agent production either in the individual being treated or in another expression system (e.g., an in vitro transcription/translation system or a host cell) from which expressed cytokine or immunological inducing agent can be obtained for administration to the individual. It is recognized that microorganisms utilized to synthesize and deliver allergenic and/or immunomodulatory proteins according to the present invention can act as an adjuvant, and that preferred microorganisms are immunostimulatory adjuvants.

Detail Description Paragraph:
[0086] In order to prolong the effect of an agent, it is often desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the agent then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of agent to polymer and the nature of the particular polymer employed, the rate of release of the agent can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides) Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body **tissues**.

Detail Description Paragraph:
[0099] Methods of encapsulating live cells are known and may also be used in accordance with the present invention for delivering antigen-secreting microorganisms to individuals. The following references are provided as examples of encapsulation of live cells. However, any method of encapsulating live cells may be used in the present invention. U.S. Pat. No. 5,084,350; U.S. Pat. No. 4,680,174; and U.S. Pat. No. 4,352,883 (all of which are incorporated herein by reference) describe the encapsulation of a prokaryotic or eukaryotic cell or cell culture in microcapsules. Briefly, U.S. Pat. Nos. 5,084,350; 4,680,174; and 4,352,883 disclose that a **tissue** sample, cell, or cell culture to be encapsulated is first prepared in finely divided form in accordance with well-known techniques and suspended in an aqueous medium suitable for maintenance and for supporting the ongoing metabolic processes of the particular cells involved. Media suitable for this purpose generally are available commercially. Thereafter, a water-soluble substance which is physiologically compatible with the cells and which can be rendered water-insoluble to form a shape-retaining coherent spheroidal mass or other shape is added to the medium. The solution is then formed into droplets containing cells together with their maintenance or growth medium and is immediately rendered water-insoluble and gelled to form shape-retaining, typically spheroidal coherent masses.

Detail Description Paragraph:
[0102] The compositions of the present invention may be employed to treat or prevent allergic reactions in a subject. Subjects are animal and human patients in need of treatment for allergies. Preferably, the animal is a domesticated mammal (e.g., a dog, a cat, a horse, a sheep, a pig, a goat, a cow, etc). Animals also include laboratory animals such as **mice**, rats, hamsters, monkeys, and rabbits. Any

individual who suffers from allergy, or who is susceptible to allergy, may be treated. It will be appreciated that an individual can be considered susceptible to allergy without having suffered an allergic reaction to the particular antigen in question. For example, if the individual has suffered an allergic reaction to a related antigen (e.g., one from the same source or one for which shared allergies are common), that individual will be considered susceptible to allergy to the relevant antigen. Similarly, if members of an individual's family are allergic to a particular antigen, the individual may be considered to be susceptible to allergy to that antigen. More preferably, any individual who is susceptible to anaphylactic shock upon exposure to food allergens, venom allergens or rubber allergens may be treated according to the present invention.

Detail Description Paragraph:
[0107] The following experiments describe the encapsulation of allergens in bacteria for use as a delivery vehicle and/or adjuvant in immunotherapy in accordance with the teachings of the present invention. Recombinant peanut allergen proteins (Ara h 1, Ara h 2, and Ara h 3; Burks et al. J Allergy Clin Immunol. 88 (2):172-9, 1991; Burks et al. J Allergy Clin Immunol. 90(6 Pt 1):962-9, 1992; Rabjohn et al. J Clin Invest. 103(4):535-42, 1999; incorporated herein by reference) were produced in E. coli BL21 cells by transforming the bacterial cells with cDNA clones encoding the proteins (see Appendix B; sequences cloned into pET24, Novagen, Madison, Wis.). The transformed cells were then injected into C3H/HEJ **mice** to determine if the allergen-expressing E. coli elicited an immune response.

Detail Description Paragraph:
[0109] The following protocol was developed for the preparation of allergen-producing E. coli cells for inoculation of **mice**.

Detail Description Paragraph:
Immune Response of **Mice**

Detail Description Paragraph:
[0124] The following protocol was utilized to determine the immune response of **mice** injected with allergen-producing bacteria. Blood was collected from the tail vein of each **mouse** used before the first injection. Enough blood was collected for antibody ELISA for each allergen and E. coli proteins. On Day Zero each **mouse** was injected with 100 microliters of the killed E. coli samples subcutaneously in the left hind flank. The **mice** were injected for the second time on Day 14 using the same procedure as Day Zero. On Day 21, a second blood sample was collected from each **mouse**. Blood samples at Day 0 and Day 21 were assayed for IgG1 and IgG2a antibodies to either Ara h 1, Ara h 2, or Ara h 3 by an ELISA assay.

Detail Description Paragraph:
[0125] **Mice** injected with E. coli producing Ara h 1 did not give detectable levels of any immunoglobulin to the Ara h 1 allergen and therefore, that data are not shown. Without limitation to theory, we speculate that this may be due to the relatively small amounts of Ara h 1 produced by these cells (see previous discussion). **Mice** injected with E. coli producing Ara h 2 contained relatively high levels of IgG1 and IgG2a. Again, without limitation to the cause, we speculated that this may be due to the amount of Ara h 2 released from these cells (see discussion above). **Mice** injected with E. coli producing Ara h 3 contained relatively high levels of IgG2a (indicative of a Th1-type response) and elicited relatively low levels of IgG1 (indicative of a Th2-type response.

Detail Description Paragraph:
[0127] The present data should be cautiously interpreted. The data in the Figures only represent O.D. levels and do not represent absolute amounts of immunoglobulin. Therefore comparisons between groups should take into consideration the data presented as O.D. However, the general trend suggests that for example, more **mice**

exhibited an IgG2a response to Ara h 3 than **mice** that exhibit an IgG1 response to Ara h 3.

CLAIMS:

4. The method of claim 1, wherein in the step of providing, the microorganism is selected from the group consisting of: E. coli, Lactococcus, **Listeria**, Vibrio, Salmonella and S. cerevisiae.

20. The composition of claim 16, wherein the microorganism is selected from the group consisting of: E. coli, Lactococcus, **Listeria**, Vibrio, Salmonella and S. cerevisiae

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DOCUMENT-IDENTIFIER: US 20030224521 A1
TITLE: Enhanced homologous recombination mediated by lambda recombination proteins

Brief Description of Drawings Paragraph:
[0019] FIG. 6 is a linear depiction of the modified defective lambda prophage as integrated on the E. coli chromosome of DY380, EL250, and EL350 cells. This figure illustrates that the defective prophages used for BAC engineering contain the X genes from cI857 to int. P.sub.L (or pL) and P.sub.R denote the lambda left and right promoters, respectively. The gam and red genes, exo and bet are under the control of P.sub.L, which is repressed by the **temperature**-sensitive repressor, cI857 at 32.degree. C. and de-repressed at 42.degree. C. tet replaces the segment from cro-bioA in DY380 cells. The araC-P.sub.BADflpe cassette or the araC-P.sub.BADcre cassette replaces the segment from cro-bioA in EL250 or EL350 cells, respectively. The **promoter of the araBAD operon (P.sub.BAD), which can be induced** by L-arabinose, controls the expression of the flpe or Cre genes. Thick black lines designate the prophage while thin lines represent E. coli sequence. < >defines the ends of the cro-bioA region that was replaced with tet, araC-P.sub.BADflpe, or araC-P.sub.BADcre.

Detail Description Paragraph:
[0044] In one embodiment, the expressed recombinase is a double strand break repair recombinase, such as lambda Beta or other single-stranded DNA binding protein; lambda Exo, or lambda Gam. In another embodiment, the extrachromosomal eukaryotic gene or gene fragment may be located on a bacterial artificial chromosome, yeast artificial chromosome, P1 artificial chromosome, plasmid or cosmid. In yet another embodiment, the eukaryotic gene or gene fragment is derived from a mammalian organism, such as a **mouse** or human.

Detail Description Paragraph:
[0047] For example, extrachromosomal DNA including the eukaryotic gene or gene fragment is introduced into a bacterial cell having an intrachromosomal gene encoding a recombinase operably linked to a de-repressible promoter. The bacterial cell is then induced to express the recombinase. A nucleic acid molecule capable of undergoing homologous recombination with the eukaryotic gene or gene fragment is

introduced into the bacterial cell. The eukaryotic gene or gene fragment undergoes homologous recombination with the nucleic acid, and altered eukaryotic gene or gene fragment may then be **isolated** and introduced into a eukaryotic cell.

Detail Description Paragraph:
[0050] A mobilizable lambda DNA is also disclosed herein that is **isolated** as a mini-lambda prophage. The mobilizable lambda DNA can be transformed into any bacterial strain of interest. The lambda DNA integrates into the bacterial chromosome to generate a defective prophage that expresses the recombinase.

Detail Description Paragraph:
[0068] In one embodiment, the de-repressible promoter is a **temperature** sensitive de-repressible promoter. For example, by increasing the **temperature**, the repressor is released from the promoter, or can no longer bind to the promoter with a high affinity, and transcription is increased from the promoter. One specific, non-limiting example is the **induction of pL promoter** activity by increasing the **temperature** of the cell. Increased **temperature** inactivates the **temperature**-sensitive repressor cI, allowing genes that are operably linked to the pL promoter to be expressed at increased levels. One of skill in the art can readily identify a repressible promoter.

Detail Description Paragraph:
[0073] Eukaryotic cell: A cell having an organized nucleus bounded by a nuclear membrane. These include lower organisms such as yeasts, slime molds, and the like, as well as cells from multicellular organisms such as invertebrates, vertebrates, and mammals. They include a variety of **tissue** types, such as, but not limited to, endothelial cell, smooth muscle cell, epithelial cell, hepatocyte, cells of neural crest origin, **tumor** cell, hematopoetic cell, immunologic cell, T cell, B cell, monocyte, macrophage, dendritic cell, fibroblast, keratinocyte, neuronal cell, glial cell, adipocyte, myoblast, myocyte, chondroblast, chondrocyte, osteoblast, osteocyte, osteoclast, secretory cell, endocrine cell, oocyte, and spermatocyte. These cell types are described in standard histology texts, such as McCormack, Introduction to Histology, (c) 1984 by J. P. Lippincott Co.; Wheater et al., eds., Functional Histology, 2nd Ed., (c) 1987 by Churchill Livingstone; Fawcett et al., eds., Bloom and Fawcett: A Textbook of Histology, (c) 1984 by William and Wilkins, all of which are incorporated by reference in their entirety. In one specific, non-limiting example, a eukaryotic cell is a stem cell, such as an embryonic stem cell.

Detail Description Paragraph:
[0075] Exogenous: The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a cell once introduced into the cell. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire chromosome **isolated** from a cell of subject X is an exogenous nucleic acid with respect to a cell of subject Y once that chromosome is introduced into Y's cell.

Detail Description Paragraph:
[0090] **Isolated: An "isolated"** biological component (such as a nucleic acid or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, and proteins. Thus, nucleic acids and proteins that have been **"isolated"** include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Detail Description Paragraph:

[0091] Knockout: Inactivation of a gene such that a functional protein product cannot be produced. A conditional knockout is a gene that is inactivated under specific conditions, such as a gene that is inactivated in a **tissue**-specific or a temporal-specific pattern. A conditional knockout vector is a vector including a gene that can be inactivated under specific conditions. A conditional knockout transgenic animal is a transgenic animal including a gene that can be inactivated in a **tissue**-specific or a temporal-specific manner.

Detail Description Paragraph:
[0109] Probes and primers: A nucleic acid probe comprises an **isolated** nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook et al., (1989) and Ausubel et al., (1997).

Detail Description Paragraph:
[0112] Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as in the case of a polymerase II type promoter, a TATA element. Enhancer and repressor elements can be located adjacent or distal to the promoter, and can be located as much as several thousand base pairs from the start site of transcription. Examples of promoters include, but are not limited to, the SV40 promoter, the CMV promoter, the .beta.-actin promoter, and **tissue**-specific promoters. Examples of **tissue**-specific promoters include, but are not limited to: probasin (which is promotes expression in prostate cells), an immunoglobulin promoter; a whey acidic protein promoter; a casein promoter; glial fibrillary acidic protein promoter; albumin promoter; .beta.-globin promoter; an insulin promoter; and the MMTV promoter. In yet another embodiment, a promoter is a hormone-responsive promoter, which promotes transcription only when exposed to a hormone. Examples of hormone-responsive promoters include, but are not limited to: probasin (which is responsive to testosterone and other androgens); MMTV promoter (which is responsive to dexamethazone, estrogen, and androgens); and the whey acidic protein promoter and casein promoter (which are responsive to estrogen).

Detail Description Paragraph:
[0118] Recombinases exert their effects by promoting recombination between two of their recombining sites. In the case of Cre, the recombining site is a Lox site (see U.S. Pat. No. 4,959,317), and-in the case of Flp the recombining site is a frt site. Similar sites are found in transposon gamma/delta, TN3, and transposon mariner. These recombining sites are comprised of inverted palindromes separated by an asymmetric sequence (Mack et al., Nuc. Acids Res. 20:4451-5, 1992; Hoess et al., Nuc. Acids Res. 14:2287-300, 1986; Kilby et al., TIG 9:413-21, 1993). Recombination between target sites arranged in parallel (so-called "direct repeats") on the same linear DNA molecule results in excision of the intervening DNA sequence as a circular molecule. Recombination between direct repeats on a circular DNA molecule excises the intervening DNA and generates two circular molecules. Both the Cre/Lox and flp/frt recombination systems have been used for a wide array of purposes such as site-specific integration into plant, insect, bacterial, yeast and mammalian chromosomes (Sauer et al., Proc. Natl. Acad. Sci. U.S.A. 85:5166-70, 1988). Positive and negative strategies for selecting or screening recombinants have been developed (Sauer et al., J. Mol. Biol. 223:911-28, 1992). The use of the recombinant systems or components thereof in transgenic **mice**, plants and insects among others reveals that hosts express the recombinase genes with no apparent deleterious effects, thus confirming that the proteins are generally well-tolerated (Orban et al., Proc. Natl. Acad. Sci. U.S.A. 89:6861-5, 1992).

Detail Description Paragraph:
[0128] Target nucleic acid sequence: The nucleic acid segment which is targeted for homologous recombination. Typically, this is a segment of chromosomal or extrachromosomal DNA in a cell. Extrachromosomal DNA harboring target nucleic acid

sequences may include episomal DNA, plasmid DNA, bacterial artificial chromosome, phagemid artificial chromosomes, yeast artificial chromosomes, cosmids, and the like. The target nucleic acid sequence usually harbors a gene or gene fragment which will be mutated in some fashion upon homologous recombination. Examples of target nucleic acid sequences include DNA sequences surrounding the tyr 145 UAG amber mutation of galK, as described in Yu et al., PNAS 97:5798-5983, 2000, and in Example 3 of this application; the second exon of **mouse** hox 1.1 gene, as described in U.S. Pat. No. 5,464,764; the human hemoglobin S gene mutation as described in Example 15 of this application.

Detail Description Paragraph:
[0132] Transgenic Animal: An animal, for example, a non-human animal such as, but not limited to, a **mouse**, that has had DNA introduced into one or more of its cells artificially. By way of example, this is commonly done by random integration or by targeted insertion. DNA can be integrated in a random fashion by injecting it into the pronucleus of a fertilized ovum. In this case, the DNA can integrate anywhere in the genome, and multiple copies often integrate in a head-to-tail fashion. There is no need for homology between the injected DNA and the host genome. In most cases, the foreign transgene is transmitted to subsequence generations in a Mendelian fashion (a germ-line transgenic).

Detail Description Paragraph:
[0147] Thus, in one example, genetic engineering steps to generate BAC recombinant include cleavage of the cassette DNA by a restriction enzyme, cleavage of target on plasmid by a restriction enzyme (wherein the vector has been pre-engineered to contain target fragments). The cassette is joined to the plasmid by DNA ligase, and the DNA is introduced into cells. Drug resistant (drug.sup.R) clones are selected, and the plasmid is **isolated**. The cloned cassette is verified and subsequently transformed into the BAC strain. Several recombination steps are used to introduce the cloned cassette into the BAC.

Detail Description Paragraph:
[0148] In contrast, in one non-limiting example, recombineering steps to generate BAC recombinants can include the generation of two primers (white and black arrows, FIG. 2B), and the generation of a PCR amplified cassette with flanking homologies. In the example pictured in FIG. 2B, exemplary striped homology segments shown are 50 base pairs long, but they can be about 100 base pairs in length, or from about 200 to about 500 base pairs in length. Phage recombination functions are induced into a BAC strain or BAC DNA is introduced into strain carrying recombination functions. The cells containing the BAC and the recombination functions are transformed with a PCR cassette. A recombinant is generated in vivo, and can then be detected by selection or counter-selection, by direct screening (colony hybridization), or by detecting a label on the nucleic acid (e.g. when DNA includes a DNA adduct or a marker such as biotin) As disclosed herein, in one specific, non-limiting example, the defective X prophage was transferred to the BAC host strain DH10B so that it can be used for BAC engineering. The modified DH10B strain called DY380 can be transformed with BAC DNA at efficiencies of 10-6 to 10-4. The utility of DY380 cells for BAC engineering has been demonstrated by introducing a 250 kbp **mouse** BAC that contains the neuronal-specific enolase 2 (Eno2) gene into DY380 cells by electroporation and then modifying the BAC by introducing a Cre-expressing targeting cassette into the 3' end of the Eno2 gene using Red recombination (see Example 20). The targeting cassette was PCR-amplified from a template plasmid using chimeric 63 nucleotide (nt) primers. The 3' 21 nucleotides of each primer was homologous to the targeting cassette, while the 5' 42 nucleotides was homologous to the last exon of Eno2 where the cassette was to be targeted (see FIG. 10). DY380 cells were then electroporated with the amplified targeting cassette and correctly targeted colonies were obtained at an efficiency approaching 10-4 following the induction of Red expression; no targeted colonies were obtained in uninduced cells.

Detail Description Paragraph:
[0149] As also disclosed herein, the modified full length BAC was purified and injected in **mouse** zygotes and a BAC transgenic line established. Two other transgenic lines carrying a shorter 25 kbp subclone of the modified Eno2 gene on pBR322 were also established as controls. The 25 kbp subclone carries the entire modified Eno2 coding region as well as 10 kbp of 5' flanking sequence and 5 kbp of 3' flanking sequence. The activity of the Cre gene in the different transgenic lines was then assessed by crossing the **mice** to ROSA26 reporter **mice**. These **mice** carry a lacZ reporter that can be activated by Cre recombinase. In **mice** carrying the full length BAC transgene, Cre activity was detected in all Eno2-positive neurons. In contrast, not all Eno2-positive neurons expressed Cre in the transgenic **mice** carrying the smaller 25 kbp subclone, and the pattern of Cre expression varied between the two different 25 kbp subclone lines. These results are consistent with previous studies showing that regulatory sequences can be located hundreds of kilobases from a gene, and highlight the usefulness of BAC engineering for in this case generating Cre-expressing lines for use in conditional knockout experiments.

Detail Description Paragraph:
[0152] Expression of a recombinase, such as, but not limited to, Cre, in **mice** carrying the cko allele catalyzes recombination between the LoxP sites and inactivates the gene. In one embodiment, transgenic animals, such as, but not limited to, transgenic **mice** (cko **mice**), can be produced including a cko allele. These **mice** allow a gene to be inactivated in a **tissue**- or temporal-specific fashion. In one specific, non-limiting example, the **mice** include a **tissue**-specific, or temporal-specific promoter operably linked to a nucleic acid encoding a recombinase. Thus, the gene of interest is inactivated when the recombinase is expressed.

Detail Description Paragraph:
[0165] In one embodiment, a linearized conditional knockout vector is introduced into embryonic stem cells. Homologous recombination can occur either upstream or downstream of the gene of interest with the inserted recombination sites to stably integrate these nucleic acid sequences into a chromosome of the embryonic stem cell. The embryonic stem cell can be used to produce a transgenic animal. Any animal can be of use in the methods disclosed herein, including human and non-human animals. A "non-human animal" includes, but is not limited to, a non-human primate, a farm animal such as swine, cattle, and poultry, a sport animal or pet such as dogs, cats, horses, hamsters, rodents, or a zoo animal such as lions, tigers, or bears. In one specific, non-limiting example, the non-human animal is a transgenic animal, such as, but not limited to, a transgenic **mouse**, cow, sheep, or goat. In one specific, non-limiting example, the transgenic animal is a **mouse**.

Detail Description Paragraph:
[0166] Advances in technologies for embryo micromanipulation permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells, such as embryonic stem cells, can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means. In one embodiment, homologous recombination is induced in an embryonic stem cell, such that an exongenous DNA is integrated into a chromosome of the embryonic stem cell. The transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. Reviews of standard laboratory procedures for the introduction of heterologous DNAs into mammalian (**mouse**, pig, rabbit, sheep, goat, cow) fertilized ova include: Hogan et al., Manipulating the **Mouse** Embryo, Cold Spring Harbor Press, 1986; Krimpenfort et al., Bio/Technology 9:86, 1991; Palmiter et al., Cell 41:343, 1985; Kraemer et al., Genetic Manipulation of the Early Mammalian Embryo, Cold Spring Harbor Laboratory Press, 1985; Hammer et al., Nature 315:680, 1985; Purcel et al., Science 244:1281, 1986; Wagner et al., U.S. Pat. No. 5,175,385; Krimpenfort et al., U.S. Pat. No. 5,175,384.

Detail Description Paragraph:
[0167] Thus, in one specific, non-limiting example, a "conditional knockout transgenic animal" is generated including the gene of interest including the two recombination sites (e.g. including two copies of recombination site 1 in a gene of interest, such as flanking an exon of a gene included in the BAC). To knockout expression of the gene of interest in the transgenic animal, a recombinase is expressed in a cell of the transgenic animal. In one specific, non-limiting example, to generate a **mouse** wherein this knockout can occur, a conditional knockout transgenic **mouse** can be mated to a second transgenic **mouse** carrying a transgene including a temporal- or **tissue**-specific promoter operably linked to a transgene encoding the recombinase. Offspring are selected that carry the gene of interest including the two recombination sites, and the gene encoding the recombinase. In these animals, the gene is knocked out in those cells wherein the recombinase is expressed.

Detail Description Paragraph:
[0180] Because homologies involved in Red-mediated recombination can be very short, targeting cassettes can also be made by simply annealing two complementary synthetic ssDNA oligonucleotides together. As described herein, a 70 bp targeting cassette constructed in this manner recombines with the E. coli chromosome to create point mutations at frequencies approaching one in a thousand electroporated cells. Point mutations corresponding to human disease-causing mutations can thus be introduced into any human or mammalian gene carried on a BAC with ease and the affect of this mutation on gene function assayed in a transgenic that carries a null mutation in the corresponding **mouse** gene.

Detail Description Paragraph:
[0182] Fragments can be subcloned from BACs by Red-mediated recombination without the use of restriction enzymes or DNA ligases. Thus, any region of the BAC is amenable to subcloning, and subcloning does not depend on the placement of appropriate restriction enzyme sites. Subcloning relies on gap repair to recombine the free ends of a linear plasmid vector with homologous sequences carried on the BAC. An example is shown in FIGS. 8 and 10. The linear plasmid vector with, for example, an amp selectable marker and an origin of replication carries the recombinogenic ends. The vector is generated, for example, by polymerase chain reaction (PCR) amplification using two chimeric primers. The 5' end of each primer has homology to the extremities of the BAC sequence to be subcloned; the 3' end of each primer is used to prime and amplify the linear plasmid DNA. Recombination generates a circular plasmid in which the DNA insert is retrieved from the BAC via gap repair. Circular recombinant plasmids are selected by their drug resistance (e.g. Amp.sup.R) phenotype. Different sizes of fragments that can be subcloned depending on the cloning vector utilized. With a high copy vector such as pBluescript, fragments up to about 25 kbp are subcloned. However, with a lower copy vector such as pBR322 is used, fragments as large as about 80 kbp can be subcloned. These larger fragments were shown to be more accurately expressed in a **tissue** specific manner (as was the entire BAC clone, see above).

Detail Description Paragraph:
[0184] As disclosed herein, recombination functions were expressed from their native location in the pL operon of a lambda prophage using the natural .lambda. repressor controls (FIG. 9). However, one limitation of the defective prophage system as disclosed in FIGS. 6 and 9 is that BACs under study must be moved into recombination-proficient DY380 cells before the BAC can be manipulated. In order to overcome this limitation, a novel prophage derivative has been generated that is **isolated** as a mini-lambda circle DNA carrying a selectable marker (e.g. a drug-resistance marker such as tet.sup.R cassette) and containing the exo, bet, and gam genes under control of the temperature inducible cI857 repressor (FIG. 12). This mini-lambda can be transformed into any bacterial cell, such as a DH10B cell that carry a BAC. The mini-lambda then integrates at the lambda attachment site to generate the defective prophage. This mobilizable prophage makes it possible to

introduce the prophage into BAC-containing DH10B libraries and obviates the need to transfer the BAC to DY380 cells.

Detail Description Paragraph:
[0188] Using the methods disclosed herein, point mutations can be introduced into a nucleic acid sequence of interest. In one specific, non-limiting example, a point mutation was engineered into the **mouse** Brca2 carried on a BAC using a 70 nt oligo. The targeting efficiency was several times higher than would be found with dsDNA created by annealing oligos and at least 50 times higher than with dsDNA generated by PCR and containing large regions of nonhomology in their center. A 140 nt oligonucleotide has also been used to introduce a 29 amino acid in-frame deletion into exon11 of the Brca2 gene and a 1.93 kb deletion into the BAC vector backbone (Swaminathan et al. Genesis 29:14-21, 2001, herein incorporated by reference). Finally, a 164 nt oligo has been used to introduce a 24 bp flag tag into the 5' end of Brca2. The targeting efficiency for the 164 nt oligo (7.7.times.10.sup.-3) was nearly the same as the targeting efficiency for generating deletions using 140 nt oligos (8.3.times.10.sup.-3 and 5.4.times.10.sup.-3, respectively).

Detail Description Paragraph:
[0203] To induce expression from the pL operon and prepare electroporation-competent cells, overnight cultures grown at 32.degree. C. from **isolated** colonies were diluted 50-fold in LB medium and were grown at 32.degree. C. with shaking to an OD.sub.600 of about 0.4-0.8. Induction was performed on a 10 ml culture in a baffled conical flask (50 ml) by placing the flask in a water bath at 42.degree. C. with shaking (200 revolutions/min) for 15 minutes. Immediately after the 15 minute induction, the flask was swirled in an ice water slurry to cool for 10 minutes. An uninduced control culture, maintained at 32.degree. C. throughout, was also placed into the ice slurry. The cooled 10 ml cultures were centrifuged for 8 minutes at 5,500.times.g at 4.degree. C. Each cell pellet was suspended in 1 ml of ice-cold sterile water, transferred to a 1.5 ml plastic microcentrifuge tube, and was spun for 20 seconds at 4.degree. C. at maximum speed in a microcentrifuge. After washing the cell pellets as described two more times, the cells were suspended in 100 .mu.l of ice cold sterile water. This volume of competent cells is sufficient for two standard electroporation reactions (.about.10.sup.8 cells per reaction). Larger cultures can be prepared for a greater number of reactions or for storage of electrocompetent cells at -80.degree. C. with 12% glycerol present. Fresh competent cells give highest efficiencies of recombination. To transform cells by electroporation, purified linear donor DNA (1 to 10 .mu.l) was mixed with competent cells in a final volume of 50 .mu.l on ice, and then pipetted into a pre-cooled electroporation cuvette (0.1 cm). The amount of donor DNA used per reaction (usually 1 to 100 ng) is indicated for relevant experiments. Electroporation was performed using a Bio-Rad Gene Pulser set at 1.8 kV, 25 .mu.F with Pulse controller of 200 ohms. Two protocols have been used interchangeably to allow segregation of recombinant from parental chromosomes within the electroporated cells. In both protocols, the electroporated cells were immediately diluted with 1 ml of LB medium. In one, the cells were incubated for 1 to 2 hours at 32.degree. C. before selecting for recombinants. In the other, the cells were immediately diluted and spread on sterile nitrocelluose filters (100 mm) on LB agar. After a 2 hour incubation at 32.degree. C., the filters were transferred to the appropriate agar plates required to select for recombinants. Aliquots were also directly spread on LB agar and incubated at 32.degree. C. to determine and examine total viable cells after electroporation. For drug resistant selection, each ml of LB medium contained 10 .mu.g of chloramphenicol, 12.5 .mu.g of tetracycline, 20 .mu.g of kanamycin, 30 .mu.g of ampicillin, or 50 g of spectinomycin.

Detail Description Paragraph:
[0204] Although recombinants were verified by more than one method, the primary detection was for an altered phenotype caused by the modified target gene. Disruption or mutation of the galK gene was confirmed by the presence of white colonies on MacConkey galactose indicator agar, disruption of the rnc gene for the

endoribonuclease RNaseIII was confirmed by the inability of lambdoid type phage to lysogenize (Court, pp. 71-116 in Belasco et al., eds., Control of Messenger RNA **Stability**, (c) 1993, Academic Press, New York), and deletion of gam, kil, and cIII in the pL operon was scored as an ability of the .lambda. lysogen to survive growth at 42.degree. C. (Court and Oppenheim; Greer, Virology 66:589-604, 1975). PCR analysis was used to confirm the altered structure caused by replacement of a gene. Southern hybridization analyses of parental and recombinant DNAs confirmed structural changes, and DNA from the recombinant clones can be amplified by PCR and sequenced.

Detail Description Paragraph:
[0246] To generate simple recombinants of pBR322 derivatives, the protocol was modified by coelectroporating the recA.sup.- strain DY331 with circular plasmid DNA (0.1 ng) and a linear drug cassette. Recombinant plasmid monomers were readily selected and **isolated**.

Detail Description Paragraph:
[0281] A modification of the method is to place DNA encoding other ssDNA binding polypeptides under control of the pL promoter. For example, the strain HME43 is further modified to delete bet and insert DNA encoding P22 Erf, RecT, or Rad52. Expression of the ssDNA binding polypeptide is induced by **temperature** shift as it is for induction of lambda bet expression. Exo and Gam, or proteins with similar function, can also be placed under control of the pL promoter. Moreover, other **inducible or constituitive promoters** can be used.

Detail Description Paragraph:
[0292] Hematopoetic stem cells from a sickle cell patient are **isolated**, cultured, and expanded ex vivo as is known in the art (Brugger, Seminars in Hematology 37[1 Suppl 2]:42-49, 2000; Dao et al., Blood 92:4612-21, 1998; Aglietta et al., Haematologica 83:824-48, 1998; Emerson, Blood 87:3082-8, 1996). A 60-mer ssDNA oligonucleotide of SEQ ID NO: 12 (AACAGACACC ATGGTGCACC TGACTCCTGA GGAGAAGTCT GCCGTTACTG CCCTGTGGGG) is synthesized and partially purified by standard techniques (Pfleiderer et al., Acta Biochimica Polonica 43:37-44, 1996; Anderson et al., Applied Biochemistry & Biotechnology 54:19-42, 1995, herein incorporated by reference).

Detail Description Paragraph:
[0293] After culture and ex vivo expansion, about 10.sup.6 hematopoetic stem cells are suspended in 0.4 mL PBS containing 0.1% glucose, about 10 .mu.M purified lambda Beta protein, and about 1 .mu.g ssDNA oligonucleotide of SEQ ID NO: 12. The cell suspension is electroporated in a 1-mL cuvette at 280V and 250.mu.F with a Gene Pulser (Bio-Rad Laboratories Inc., Hercules, Calif., USA). Cells are then plated and cultured. Homologous recombinants harboring the mutation are identified and clonally **isolated**, further expanded ex vivo, and may be returned to the patient, or cultured for additional in vitro study.

Detail Description Paragraph:
[0295] Other types of stem cells can be used to correct the specific gene defects associated with cells derived from such stem cells. Such other stem cells include epithelial, **liver**, **lung**, muscle, endothelial, mesenchymal, neural and bone stem cells.

Detail Description Paragraph:
[0298] For example, sequences encoding positive selection marker neomycin resistance gene are synthesized as a series of overlapping 70-mer oligonucleotides, 20 base pairs of overlap and 3' overhangs. The 3' terminal oligonucleotides are designed to insert into the second exon of the **mouse** hox 1.1 gene as described in U.S. Pat. No. 5,464,764. Because the overlapping oligonucleotides combine to encode a promoterless neomycin resistance gene, only those that successfully incorporate into the targeted **mouse** hox 1.1 second exon will express the neo gene product and

have the neomycin resistance phenotype. The targeting is designed to provide the synthetic neomycin resistance gene with an operable promoter and translation start derived from the **mouse** hox 1.1 gene. The targeting DNA is also designed so that random incorporations elsewhere in the ES cell genome are unlikely to be operably linked to any promoter to allow transcription and translation.

Detail Description Paragraph:
[0305] Preferably, DNA designed for homologous recombination with a target DNA sequence in plants are combined with lambda Beta protein or other ssDNA protein and directly transferred to plant protoplasts by way of methods analogous to that previously used to introduce transgenes into protoplasts. Concentration of the DNA and ssDNA binding proteins are as described in Example 15 (see, e.g. Paszkowski et al., EMBO J., 3:2717-2722, 1984; Hain et al., Mol. Gen. Genet., 199, 161-168, 1985; Shillito et al. Bio./Technology 3:1099-1103, 1985; and Negrutiu et al., Plant Mol. Bio. 8:363-373, 1987). Alternatively, the PNS vector is contained within a liposome which can be fused to a plant protoplast (see, e.g. Deshayes et al., EMBO J. 4:2731-2738, 1985) or is directly inserted to plant protoplast by way of intranuclear microinjection (see, e.g. Crossway et al., Mol. Gen Genet. 202:179-185, 1986, and Reich et al., Bio/Technology 4:1001-1004, 1986). Microinjection can be used for transfecting protoplasts. The DNA and ssDNA binding proteins can also be microinjected into meristematic inflorescences. De la Pena et al., Nature 325:274-276, 1987. Finally, **tissue** explants can be transfected by way of a high velocity microprojectile coated with the DNA and ssDNA binding proteins analogous to the methods used for insertion of transgenes (see, e.g. Vasil, Bio/Technology 6:397, 1988; Klein et al., Nature 327:70, 1987; Klein et al., Proc. Natl. Acad. Sci. U.S.A. 85:8502, 1988; McCabe et al., Bio/Technology 6:923, 1988; and Klein et al., Genetic Engineering, Vol 11, J. K. Setlow editor (Academic Press, N.Y., 1989)). Such transformed explants can be used to regenerate for example various serial crops. Vasil, Bio/Technology 6:397, 1988.

Detail Description Paragraph:
[0306] Once the DNA and ssDNA binding protein have been inserted into the plant cell by any of the foregoing methods, homologous recombination targets the oligonucleotide to the appropriate site in the plant genome. As in previous examples, the oligonucleotide may be a series of overlapping ssDNAs with 5' or 3' overhangs. Depending upon the methodology used to transfect, selection is performed on **tissue** cultures of the transformed protoplast or plant cell. In some instances, cells amenable to **tissue** culture may be excised from a transformed plant either from the F0 or a subsequent generation.

Detail Description Paragraph:
[0325] Production of transgenic **mice**. Modified BAC and the p25-kbp subclone DNAs were purified using cesium chloride gradients as described (Antoch et al., 1997). The 25-kbp subclone DNA was linearized by NotI digestion before microinjection. BAC DNA (1 .mu.g/ml) and 25-kbp subclone DNA (2 .mu.g/ml) were microinjected into the pronucleus of (C3H/HeN-Mtv.sup.- X C57BL/6Ncr) F.sub.2 zygotes. Transgenic founders were subsequently identified by Southern analysis using a Cre probe or by PCR using primers 5'CTGCTGGAAGATGGCGATTCTCG (SEQ ID NO: 36) and 5'AACAGCAGGAGCGGTGAGTC (SEQ ID NO: 37) that flank the 3' insertional junction.

Detail Description Paragraph:
[0326] Histochemical analysis of .beta.-galactosidase expression. **Mice** at 4 to 5 weeks of age were sacrificed in CO.sub.2 and perfused with 4% paraformaldehyde in PBS (pH 7.3). The **brains**, spinal cords and eyes were removed and postfixed for 3 hours. Vibratome sections (20 .mu.m) of **brains** were mounted on slides and used directly for X-gal staining or for immunocytochemistry. For spinal cords and eyes, cryostat sections (20 .mu.m) were used that were made by cryoprotecting **tissues** in 30% sucrose in PBS overnight and embedding the **tissues** in freezing compound (OCT, Sakura). Before X-gal staining, samples on slides were postfixed with 0.25% glutaraldehyde in PBS and briefly washed with rinse solution (0.1 phosphate buffer

pH7.3, 0.1% deoxycholic acid, 0.2% NP40 and 2 mM MgCl.sub.2). X-gal staining was performed by incubating samples in staining buffer (2.5 mg/ml X-gal, 5mM potassium ferricyanide and 5 mM potassium ferrocyanide in staining buffer) for 2 hours at 37.degree. C. followed by counterstaining with 0.25% eosin (Fisher).

Detail Description Paragraph:
[0341] The defective prophage DNA can be **isolated** and purified from these lysogens, if after a 15 minute induction, cells are lysed and DNA is **isolated** by plasmid purification protocols, i.e. by Qiagen columns. The circular phage DNA with its drug markers can be purified. This DNA cannot replicate upon retransfection into E. coli strains but it can express its pL operon and Int function to allow integration of the circular DNA by site specific recombination between attP in the circular DNA and attB in the bacterial chromosome. Only Int and the host IHF functions are required for site-specific recombination. Such integrated DNAs are stable, are immune and can be selected by the drug marker each carries.

Detail Description Paragraph:
[0345] To test the prophage system of Example 17 in BAC engineering, the efficiency of BAC recombination in EL250 cells was investigated. In the experiments described in this example, a selectable cassette was targeted to a **mouse** neuron-specific locus in a 250 kb BAC. The BAC was then further modified to enhance its usefulness in subsequent **mouse** genetic studies. These experiments validated an improved strategy and provided improved reagents for BAC engineering using the lambda recombination system.

Detail Description Paragraph:
[0351] Generating and **Isolating** a BAC With a Disrupted Eno2 Locus

Detail Description Paragraph:
[0362] Upon removal of the undesirable LoxP site, the modified 284H12 BAC was used in the transgenic **mouse** studies described in Example 21.

Detail Description Paragraph:
[0371] Production of Transgenic **Mice** Using BACs

Detail Description Paragraph:
[0372] Examples 18-20 describe the construction of a modified BAC believed to contain all of the regulatory sequences needed for neural-specific Cre expression in transgenic **mice**. To investigate this hypothesis, the modified BAC described in Example 18 was injected into (C3H/HeN-Mtv.sup.- X C57BL/6Ncr) F.sub.2 zygotes. A BAC transgenic line carrying approximately two copies of the transgene was then established.

Detail Description Paragraph:
[0374] The transgenic **mice** were crossed to ROSA26 reporter **mice**, which contain a lacZ reporter that can be activated by Cre recombinase (Soriano, Nature Genetics 221:70-71, 1999). Double heterozygotes were subsequently analyzed by X-gal staining at 4 weeks of age.

Detail Description Paragraph:
[0375] Several different **tissues** were examined for X-gal expression including the **brain**, spinal cord, eye, **lung**, heart, intestine, muscle, **liver**, **spleen**, and kidney. Blue stained cells were found only in neural **tissue** in the three transgenic lines, indicating that both the BAC and the 25-kbp subclone contain the regulatory elements needed for neural-specific expression. The pattern of Cre activity was, however, different in the three lines. Vibratome sections of the **brain** from the BAC transgenic **mice** showed blue-stained cells throughout the gray matter but not in the white matter, indicative of Cre activity in most neurons but not in glial cells. In contrast, X-gal staining in the 25 kbp-1 and 25 kbp-2 transgenic **mice** was present in only a subset of neurons and expression was variable between the two different

lines.

Detail Description Paragraph:
[0376] Higher power magnification of the cerebellum of the BAC transgenic **mice** showed that Cre was expressed in virtually all neuronal cells. This included Purkinje cells in the Purkinje cell layer, granule and Golgi cells in the granular layer, basket cells and stellate cells in the molecular layer and neurons of the deep cerebellar nuclei. In contrast, in the 25 kbp-1 line, Cre was expressed in only a subset of Golgi cells in addition to a few cells in the granule and Purkinje cell layers. Glial cells of white matter also expressed Cre indicative of leaky expression. In the 25 kbp-2 line, Cre expression was limited to the gray matter and included a variety of neuronal cell types, including most basket cells, stellate cells, Purkinje cells and neurons of the deep cerebellar nuclei. In contrast, few granule cells and Golgi cells in the granule layer expressed Cre.

Detail Description Paragraph:
[0377] Higher power magnification of the hippocampus and cortex showed similar results. In the hippocampus of BAC transgenic **mice**, virtually all neurons in the cornu Ammonis (CA) region and the dentate gyrus (DG) expressed Cre. The same was true in the cortex, where all six layers of the cortex that contained neurons (layers II-VI) expressed Cre. In contrast, the hippocampus of 25 kbp-1 transgenic **mice** showed reduced Cre expression in the DG (FIG. 4E) and layers II and III of cortex. The 25 kbp-2 transgenic **mice** showed even lower levels of Cre expression in the DG. The CA1 and CA2 regions of the CA also failed to express Cre. Cre expression was also greatly reduced in the cortex, with layers II and III showing most the reduction.

Detail Description Paragraph:
[0378] Cre activity in the spinal cord, dorsal root ganglion (DRG) and retina of the transgenic **mice** was also examined in order to determine whether Cre was expressed in mature neurons within the peripheral nervous system. Similar to what was observed for the central nervous system, Cre was expressed in most mature peripheral neurons in the BAC transgenic **mice** while fewer peripheral neurons expressed Cre in the two 25 kbp transgenic lines.

Detail Description Paragraph:
[0379] To determine whether Cre was expressed in all Eno2 protein-positive neurons, a section from the **brain** of a BAC transgenic animal was immunostained with an anti-Eno2 antibody followed by X-gal staining for Cre activity. Virtually all Eno2-positive neurons were active for Cre. Thus, Cre expression in BAC transgenic animals correlated tightly with native **mouse** Eno2 promoter-enhancer activity.

Detail Description Paragraph:
[0381] The ability to precisely manipulate large fragments of genomic DNA, independent of the location of appropriate restriction enzyme sites, has many applications for functional genomics, both in the **mouse** and in other organisms. As shown herein, Cre can be introduced into the coding regions of genes carried on BACs facilitating the generation of Cre-expressing transgenic lines for use in conditional knockout studies or for use in conditional gene expression studies. Genes can also be epitope tagged and microinjected into the germline of **mice** carrying a mutation in the gene. If the epitope tagged transgene rescues the mutant phenotype, the epitope tagged protein is functional and the epitope tag can serve as a marker for expression of the gene. Likewise, a gene carried on a BAC can be replaced with another gene and the function of the "knock-in" mutation assayed in transgenic **mice**.

Detail Description Paragraph:
[0388] As unequivocally demonstrated in this example, the surprisingly high recombination efficiency offered by this recombination system makes it possible to manipulate BAC or other DNA without drug selection. Point mutations, deletions, or

insertions can now be engineered into any gene on a BAC in the absence of a confounding linked drug selection marker or a LoxP or frt site. In cases where the gene is mutated in human disease, the exact disease-causing mutations can be engineered on the BAC and the effect of these mutations analyzed in transgenic **mice**.

Detail Description Paragraph:
[0402] Gene Targeting in **Mouse** ES Cells : 20 ug NotI-linearized Evi9 cko-targeting vector (PL460) DNA was electroporated into 10.times.10.sup.6 CJ7 ES cells that were growing on mitomycin-C-inactivated STO cells. Transfectanis were selected in M15 medium (15% fetal bovine serum in DMEM with 2 mM L-glutamine) with G418 (180 .mu.g/ml) and ganciclovir (2 .mu.M). Targeted clones were identified on Southern blots with the 5' and 3' probes.

Detail Description Paragraph:
[0406] In order to make subcloning by GAP repair possible, a BAC must be first transferred from its strain of origin (DH10B) into an E. coli strain that contains exo, bet, and gam. In the experiments described herein, BACs are transferred into EL350 E. coli cells (Examples 20-21). EL350 cells were made by constructing a defective lambda prophage in DH10B cells, to create DY380 cells (Example 18) since DH10B is one of the few E. coli strains known that can be efficiently transformed with BAC DNA. A Cre gene under the control of the arabinose **inducible promoter**, P.sub.BAD, was then introduced into the defective prophage carried in DY380 cells, to produce EL350 cells (Lee et al., Genomics 73:56-65, 2001). In EL350 cells, the homologous recombination functions encoded by the red genes can be controlled by **temperature**, while the Cre gene can be controlled by arabinose. As disclosed herein, it is much easier to transform electro-competent EL350 or DY380 cells produced from overnight cultures, than from exponentially growing cells. When BAC DNA is electroporated into stationary electro-competent cells and the BAC-containing cells selected using the chloramphenicol resistance (Cam.sup.r) gene that is carried in the BAC vector backbone, 100 to 1000 Cam.sup.r colonies are routinely obtained from 50 ng of BAC DNA, and virtually all of the colonies contain unrearranged BACs. A complete list of the reagents used in these studies can be found in Table 9.

Detail Description Paragraph:
[0416] To overcome these problems, a new selection cassette (PL451) was constructed. PL451 was constructed by introducing a frt site upstream of Neo, and frt and LoxP sites downstream of Neo, in PGKNeobpA, a selection cassette that is commonly used for gene targeting in ES cells (FIG. 16A). Similar to PL452, a bacterial EM7 promoter was introduced in between the PGK promoter and the coding sequence of Neo. This selection cassette works efficiently in both E. coli and **mouse** ES cells. frt is the DNA recognition site for Flp recombinase. DNA located between two frt sites in **mouse** ES cells can be excised by transient expression of a genetically enhanced Flp recombinase (Flpe) (Buchholz et al., Nat Biotechnol 16:657-662 1998), that works well in ES cells. In this case, single frt and single LoxP sites, were left behind at the targeted locus (FIG. 16A). Only one Flpe recombination product is possible, which ensures that all excision products are the correct ones. Alternatively, the PL451 selection cassette can be removed after the conditional allele is introduced into the **mouse** germ line by breeding the **mice** to one of the **mouse** strains that expresses Flpe in the **mouse** germ line (Rodriguez et al., 2000). Subsequent expression of Cre recombinase will excise the entire DNA between the LoxP sites located on either side of Evi9 exon 4, and create an Evi9 null allele. Cre can be expressed in the **mouse** germ line to create a germ line null allele, or in somatic cells.

Detail Description Paragraph:
[0421] In order to use high copy plasmids such as pBluescript for vector construction, modifications were made in the way the .lambda. Red system was used. For example, co-electroporation was used to target the floxed Neo cassette to the

plasmid, instead of introducing the Neo cassette into cells that already carried the plasmid. Induction of the .lambda. Red genes into cells that carry multiple plasmids can cause the formation of plasmid complexes due to rolling-circle replication (Feiss et al., Gene 17:123-130, 1992). Co-transformation of the Neo cassette and the plasmid minimizes this problem, but still provides a high enough frequency of homologous recombination to generate the targeted plasmid. Cre-expressing EL350 cells were also used to excise the floxed Neo cassette from the targeted plasmid. When multiple plasmid molecules containing LoxP sites are present in a cell expressing Cre, intermolecular recombination between the LoxP sites can occur, resulting in plasmid loss. Electroporation of a small amount of plasmid DNA containing the floxed Neo cassette into Cre-expressing EL350 cells avoids this problem, yet still allows for the efficient excision of the Neo cassette. Two new selection cassettes (loxP-PGK-EM7-NeobpA-loxP and FRT-PGK-EM7-NeobpA-FRT-- loxP) were also constructed that worked well in both E. coli and **mouse** ES cells. The second selection cassette contains two frt sites and one LoxP site that flank the selection cassette. This makes it possible to remove this selection cassette following homologous recombination in ES with Flpe recombinase, leaving behind frt-LoxP sites at the targeted locus.

Detail Description Paragraph:
[0422] Additionally, 200-500 bp homology arms that contain SINE, LINE or short DNA repeats such as CA repeats have been used for retrieving and targeting. Efficient recombination was still achieved in all cases. In some circumstances, longer homology arms can help in avoiding problems created by sequencing errors in the public databases, or strain polymorphisms. This can be of use when modifying human DNA where polymorphisms are common. With its high efficiency and reliability, more than ten cko-targeting vectors have been constructed. Four of the cko-targeting vectors have been introduced into ES cells for homologous recombination. All four targeting constructs gave rise to highly efficient gene targeting frequencies in **mouse** ES cells: the frequency of cko alleles ranged from 20 to 40% of the G418.sup.r, Ganc.sup.r colonies.

CLAIMS:

15. The method of claim 9, wherein the single stranded DNA binding polypeptide is lambda Beta, and the cell further comprises a nucleic acid encoding Gam and a protein selected from the group consisting of Exo, Arabidopsis F12A21.16, cholera virus orf, B. subtilis YqaJ gene, **Listeria** phage A 118 gp47, B. subtilis phage SPP1 gene 34.1, and African swine virus orf operably linked to the de-repressible promoter.

123. The method of claim 120, wherein the transgenic animal is a transgenic **mouse**.

4. Document ID: US 20030153527 A1

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DOCUMENT-IDENTIFIER: US 20030153527 A1
TITLE: Method for introducing and expressing genes in animal cells, and live invasive bacterial vectors for use in the same

Summary of Invention Paragraph:
[0001] The present invention relates to a method for introducing endogenous or foreign genes into animal cells using live invasive bacteria as vectors. The method allows for the delivery of eukaryotic expression cassettes encoding the endogenous or foreign genes into animal cells or animal **tissue**, and is useful for expressing, e.g., vaccine antigens, therapeutic agents, immunoregulatory agents, antisense RNAs, and catalytic RNAs, in animal cells or animal **tissue**.

Summary of Invention Paragraph:
[0003] The advent of recombinant DNA technology has greatly accelerated the development of vaccines to control epidemic, endemic, and pandemic infectious diseases (Woodrow et al, New Generation Vaccines: The Molecular Approach, Eds., Marcel Dekker, Inc., New York, N.Y. (1989); Cryz, Vaccines and Immunotherapy, Ed., Pergamon Press, New York, N.Y. (1991); and Levine et al, Ped. Ann., 22:719-725 (1993)). In particular, this technology has enabled the growth of a new class of vaccines called bacterial vector vaccines (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, N.Y., pages 161-188 and 269-288 (1989); and Mims et al, In: Medical Microbiology, Eds., Mosby-Year Book Europe Ltd., London (1993)). These vaccines can enter the host, either orally, intranasally or parenterally. Once gaining access to the host, the bacterial vector vaccines express an engineered prokaryotic expression cassette contained therein that encodes a foreign antigen(s). Foreign antigens can be any protein (or part of a protein) or combination thereof from a bacterial, viral, or parasitic pathogen that has vaccine properties (New Generation Vaccines: The Molecular Approach, supra; Vaccines and Immunotherapy, supra; Hilleman, Dev. Biol. Stand., 82:3-20 (1994); Formal et al, Infect. Immun. 34:746-751 (1981); Gonzalez et al, J. Infect. Dis., 169:927-931 (1994); Stevenson et al, FEMS Lett., 28:317-320 (1985); Aggarwal et al, J. Exp. Med., 172:1083-1090 (1990); Hone et al, Microbial. Path., 5:407-418 (1988); Flynn et al, Mol. Microbiol., 4:2111-2118 (1990); Walker et al, Infect. Immun., 60:4260-4268 (1992); Cardenas et al, Vacc., 11:126-135 (1993); Curtiss et al, Dev. Biol. Stand., 82:23-33 (1994); Simonet et al, Infect. Immun., 62:863-867 (1994); Charbit et al, Vacc., 11:1221-1228 (1993); Turner et al, Infect. Immun., 61:5374-5380 (1993); Schodel et al, Infect. Immun., 62:1669-1676 (1994); Schodel et al, J. Immunol., 145:4317-4321 (1990); Stabel et al, Infect. Immun., 59:2941-2947 (1991); Brown, J. Infect. Dis., 155:86-92 (1987); Doggett et al, Infect. Immun., 61:1859-1866 (1993); Brett et al, Immunol., 80:306-312 (1993); Yang et al, J. Immunol., 145:2281-2285 (1990); Gao et al, Infect. Immun., 60:3780-3789 (1992); and Chatfield et al, Bio/Technology, 10:888-892 (1992)). Delivery of the foreign antigen to the host **tissue** using bacterial vector vaccines results in host immune responses against the foreign antigen, which provide protection against the pathogen from which the foreign antigen originates (Mims, The Pathogenesis of Infectious Disease, Academic Press, London (1987); and New Generation Vaccines: The Molecular Approach, supra).

Summary of Invention Paragraph:
[0019] Successful delivery of DNA to animal **tissue** has been achieved by cationic liposomes (Watanabe et al, Mol. Reprod. Dev., 38:268-274 (1994)), direct injection of naked DNA into animal muscle **tissue** (Robinson et al, Vacc., 11:957-960 (1993); Hoffman et al, Vacc., 12:1529-1533; (1994); Xiang et al, Virol., 199:132-140 (1994); Webster et al, Vacc., 12:1495-1498 (1994); Davis et al, Vacc., 12:1503-1509 (1994); and Davis et al, Hum. Molec. Gen., 2:1847-1851 (1993)), and embryos (Naito et al, Mol. Reprod. Dev., 39:153-161 (1994); and Burdon et al, Mol. Reprod. Dev., 33:436-442 (1992)), or intradermal injection of DNA using "gene gun" technology (Johnston et al, supra). A limitation of these techniques is that they only efficiently deliver DNA to parenteral sites. At present, effective delivery of eukaryotic expression cassettes to mucosal **tissue** has been met with limited success. This is presumably due to poor access to these sites, toxicity of the delivery vehicles or instability of the delivery vehicles when delivered orally.

Summary of Invention Paragraph:

[0021] The delivery of endogenous and foreign genes to animal **tissue** for gene therapy has shown significant promise in experimental animals and volunteers (Nabel, Circulation, 91:541-548 (1995); Coovert et al, Curr. opin. Neuro., 7:463-470 (1994); Foa, Bill. Clin. Haemat., 7:421-434 (1994); Bowers et al, J. Am. Diet. Assoc., 95:53-59 (1995); Perales et al, Eur. J. Biochem., 226:255-266 (1994); Danko et al, Vacc., 12:1499-1502 (1994); Conry et al, Canc. Res., 54:1164-1168 (1994); and Smith, J. Hemat., 1:155-166 (1992)). Recently, naked DNA vaccines carrying eukaryotic expression cassettes have been used to successfully immunize against influenza both in chickens (Robinson et al, supra) and ferrets (Webster et al, Vacc., 12:1495-1498 (1994)); against Plasmodium yoelii in **mice** (Hoffman et al, supra); against rabies in **mice** (Xiang et al, supra); against human carcinoembryonic antigen in **mice** (Conry et al, supra) and against hepatitis B in **mice** (Davis et al, supra). These observations open the additional possibility that delivery of endogenous and foreign genes to animal **tissue** could be used for prophylactic and therapeutic applications.

Summary of Invention Paragraph:
[0022] Therefore, there is a need to deliver eukaryotic expression cassettes, encoding endogenous or foreign genes that are vaccines or therapeutic agents to animal cells or **tissue**. In particular, a method that delivers eukaryotic expression cassettes to mucosal surfaces is highly desirable. Bacterial vector vaccines have been used in the past to deliver foreign antigens encoded on prokaryotic expression cassettes to animal **tissue** at mucosal sites.

Summary of Invention Paragraph:
[0023] The present invention describes a novel and unexpected finding that invasive bacteria are capable of delivering eukaryotic expression cassettes to animal cells and **tissue**. An important aspect of using live invasive bacteria to deliver eukaryotic expression cassettes is that they are capable of delivering DNA to mucosal sites.

Summary of Invention Paragraph:
[0024] Heretofore, there has been no documented demonstration of live bacteria invading animal cells and introducing a eukaryotic expression cassette(s), which then is expressed by the infected cells and progeny thereof. That is, the present invention provides the first documentation of genetic exchange between live invasive bacteria and animals cells. Heretofore, foreign antigen delivery by live bacterial vector vaccines merely involved delivery of prokaryotic expression cassettes to and expression of the foreign antigen by the bacterial vaccine vector, in animal cells or **tissues**. In contrast, the present invention involves the delivery of eukaryotic expression cassettes by live bacterial strains to animal cells in vitro or to cells in animal **tissue**, and expression of the eukaryotic expression cassettes by the animal cell or cells in animal **tissue**.

Summary of Invention Paragraph:
[0025] An object of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes to animal cells or animal **tissue**.

Summary of Invention Paragraph:
[0026] Another object of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes encoding a vaccine antigen(s) to animal cells or animal **tissue**.

Summary of Invention Paragraph:
[0027] Another object of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes encoding therapeutic agents to animal cells or animal **tissue**.

Summary of Invention Paragraph:

[0028] Yet another objective of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes encoding biologically active RNA species to animal cells or animal **tissue**.

Detail Description Paragraph:
[0036] Animal cells are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxanomic position lies within the kingdom animalia. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular **tissue** source of the cells is not critical to the present invention.

Detail Description Paragraph:
[0050] As used herein, "invasive bacteria" are bacteria that are capable of delivering eukaryotic expression cassettes to animal cells or animal **tissue**. "Invasive bacteria" include bacteria that are naturally capable of entering the cytoplasm or nucleus of animal cells, as well as bacteria that are genetically engineered to enter the cytoplasm or nucleus of animal cells or cells in animal **tissue**.

Detail Description Paragraph:
[0063] The attenuating mutations can be either constitutively expressed or under the control of **inducible promoters, such as the temperature** sensitive heat shock family of promoters (Neidhardt et al, supra), or the anaerobically **induced nirB promoter** (Harborne et al, Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as uapA (Gorfinkiel et al, J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer et al, J. Bact., 176:6159-6164 (1994)).

Detail Description Paragraph:
[0100] Alternatively, any bacteria could be genetically engineered to mimic mucosal **tissue** tropism and invasive properties, as discussed above, that thereby allow said bacteria to invade mucosal **tissue**, and deliver genes at those sites.

Detail Description Paragraph:
[0101] It is also possible to change the **tissue** specificity of the invasive bacteria by expression of a gene product singularly or in combination, e.g., the Plasmodium vivax reticulocyte binding proteins-1 and -2 bind specifically to erythrocytes in humans and primates (Galinski et al, Cell, 69:1213-1226 (1992)); Yersinia Invasin recognizes .beta.1 integrin receptors (Isberg et al, Trends Microbiol., 2:10-14 (1994)); asialoorosomucoid is a ligand for the asilogycoprotein receptor on hepatocytes (Wu et al, J. Biol. Chem., 263:14621-14624 (1988)); presence of insulin-poly-L-lysine has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz et al, Expt. Cell Res., 199:323-329 (1992)); p60 of Listeria monocytogenes allows for tropism for hepatocytes (Hess et al, Infect. Immun., 63:2047-2053 (1995)) and Trypanosoma cruzi expresses a 60 kDa surface protein which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria et al, Cell, 67:411-421 (1991)).

Detail Description Paragraph:
[0104] These cassettes usually are in the form of plasmids, and contain various promoters well-known to be useful for driving expression of genes in animal cells, such as the viral derived SV40, CMV and, RSV promoters or eukaryotic derived .beta.-casein, uteroglobin, .beta.-actin or tyrosinase promoters. The particular promoter is not critical to the present, except in the case where the object is to obtain expression in only selective cell types. In this case, the promoter is selected to be one which is only active in the selected cell type. Examples of **tissue** specific promoters include, but are not limited to, .alpha. S1- and .beta.-casein promoters which are specific for mammary **tissue** (Platenburg et al, Trans. Res., 3:99-108 (1994); and Maga et al, Trans. Res., 3:36-42 (1994)); the phosphoenolpyruvate carboxykinase promoter which is active in **liver**, kidney,

adipose, jejunum and mammary **tissue** (McGrane et al, J. Reprod. Pert., 41:17-23 (1990)); the tyrosinase promoter which is active in **lung and spleen** cells, but not testes, **brain**, heart, **liver** or kidney (Vile et al, Canc. Res., 54:6228-6234 (1994)); the involucerin promoter which is only active in differentiating keratinocytes of the squamous epithelia (Carroll et al, J. Cell Sci., 103:925-930 (1992)); and the uteroglobin promoter which is active in **lung** and endometrium (Helftenbein et al, Annal. N.Y. Acad. Sci., 622:69-79 (1991)).

Detail Description Paragraph:
[0105] Alternatively, cell specific enhancer sequences can be used to control expression, for example human neurotropic papovirus JCV enhancer regulates viral transcription in glial cells alone (Remenick et al, J. Virol., 65:5641-5646 (1991)). Yet another way to control **tissue** specific expression is to use a hormone responsive element (HRE) to specify which cell lineages a promoter will be active in, for example, the MMTV promoter requires the binding of a hormone receptor, such as progesterone receptor, to an upstream HRE before it is activated (Beato, FASEB J., 5:2044-2051 (1991); and Truss et al, J. Steroid Biochem. Mol. Biol., 41:241-248 (1992)).

Detail Description Paragraph:
[0107] In the present invention, the live invasive bacteria can deliver eukaryotic expression cassettes encoding a gene into an animal cell or animal **tissue**. The gene may be either a foreign gene or a endogenous gene. As used herein, "foreign gene" means a gene encoding a protein or fragment thereof or anti-sense RNA or catalytic RNA, which is foreign to the recipient animal cell or **tissue**, such as a vaccine antigen, immunoregulatory agent, or therapeutic agent. An "endogenous gene" means a gene encoding a protein or part thereof or anti-sense RNA or catalytic RNA which is naturally present in the recipient animal cell or **tissue**.

Detail Description Paragraph:
[0117] In the present invention, the live invasive bacteria can also deliver eukaryotic expression cassettes encoding a therapeutic agent to animal cells or animal **tissue**. For example, the eukaryotic expression cassettes can encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof. Alternatively, the eukaryotic expression cassettes can encode synthetic genes, which encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof.

Detail Description Paragraph:
[0118] Examples of **tumor** specific antigens include prostate specific antigen (Gattuso et al, Human Pathol., 26:123-126 (1995)), TAG-72 and CEA (Guadagni et al, Int. J. Biol. Markers, 9:53-60 (1994)), MAGE-1 and yrosinase (Coulie et al, J. Immunothera., 14:104-109 (1993)). Recently it has been shown in **mice** that immunization with non-malignant cells expressing a **tumor** antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant **tumor** cells displaying the same antigen (Koeppen et al, Anal. N.Y. Acad. Sci., 690:244-255 (1993)).

Detail Description Paragraph:
[0120] Examples of autoimmune antigens include IAS .beta. chain (Topham et al, Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). Vaccination of **mice** with an 18 amino acid peptide from IAS .beta. chain has been demonstrated to provide protection and treatment to **mice** with experimental autoimmune encephalomyelitis (Topham et al, supra).

Detail Description Paragraph:
[0121] Alternatively, in the present invention, live invasive bacteria can deliver eukaryotic expression cassettes encoding immunoregulatory molecules. These immunoregulatory molecules include, but are not limited to, growth factors, such as M-CSF, GM-CSF; and cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 or IFN-.gamma.. Recently, delivery of cytokines expression cassettes to **tumor tissue** has

been shown to stimulate potent systemic immunity and enhanced **tumor** antigen presentation without producing a systemic cytokine toxicity (Golumbek et al, Canc. Res., 53:5841-5844 (1993); Golumbek et al, Immun. Res., 12:183-192 (1993); Pardoll, Curr. Opin. Oncol., 4:1124-1129 (1992); and Pardoll, Curr. Opin. Immunol., 4:619-623 (1992)).

Detail Description Paragraph:
[0123] In the present invention, live invasive bacteria can also deliver eukaryotic expression cassettes encoding proteins to animal **tissue** from which they can later be harvested or purified. An example is the delivery of a eukaryotic expression cassette under the control of a mammary specific viral promoter, such as derived from **mouse** mammary **tumor** virus (ATCC No. VR731), encoding .alpha..sub.1-antitrypsin to mammary **tissue** of a goat or sheep.

Detail Description Paragraph:
[0124] Alternatively an invasive bacteria carrying a eukaryotic expression cassette can be introduced to a **tissue** site such that it would not spread from such a site. This could be accomplished by any of several methods including delivery of a very limited dose, delivery of a severely attenuated auxotrophic strain, such as an asd mutant (Curtiss et al, supra) that will be rapidly inactivated or die, or delivery of a bacterial strain that contains attenuating lesions, such as a suicide systems (Rennell et al, supra; and Reader et al, supra) under the control of a strong promoter, such as the anerobic nirB promoter (Harborne et al, supra) which will be switched on within the recipient host **tissue**. Additionally, through use of different species and/or serotypes multiple doses of invasive bacteria, the eukaryotic expression cassette of interest can be given to an animal so as to manipulate expression levels or product type. This approach obviates the need for specially raised transgenic animals containing **tissue** specific promoters and having tight control of expression, as is currently the case (Janne et al, Int. J. Biochem., 26:859-870 (1994); Mullins et al, Hyperten., 22:630-633 (1993); and Persuy et al, Eur. J. Bichem., 205:887-893 (1992)).

Detail Description Paragraph:
[0125] As a further alternative, single or multiple eukaryotic expression cassettes encoding **tumor**-specific, transplant, and/or autoimmune antigens, can be delivered in any single or multiple combination with eukaryotic expression cassettes encoding immunoregulatory molecules or other proteins.

Detail Description Paragraph:
[0127] The invasive bacteria containing the eukaryotic expression cassette can be used to infect animal cells that are cultured in vitro. The animal cells can be further cultured in vitro, and the cells carrying the desired genetic trait can be enriched by selection for or against any selectable marker introduced to the recipient cell at the time of bactofection. Such markers may include antibiotic resistance genes; e.g., hygromycin, or neomycin, selectable cell surface markers, or any other phenotypic or genotypic element introduced or altered by bactofection. These in vitro-infected cells or the in vitro-enriched cells can then be introduced into animals intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host **tissue**.

Detail Description Paragraph:
[0140] HeLa cells (ATCC No. CCL-2) were grown on plastic **tissue** culture plates at 37.degree. C. in 5% (v/v) CO.sub.2 in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2.0 mM L-glutamine, 1.0 mM L-pyruvate, 50 U/ml penicillin and 50 .mu.g/ml streptomycin (hereinafter "RPMI/FBS"). 24 to 48 hours prior to bactofection, the HeLa cells were trypsinized with 0.25% (w/v) trypsin containing 1.0 mM EDTA, and split by limiting dilution such that they were 40-60% confluent at the time of the experiment.

Detail Description Paragraph:

[0156] Thus, it is evident that the method of the present invention is not restricted to one animal cell type, but is applicable to animal cells derived from various **tissues**.

Detail Description Paragraph:
Delivery of a Reporter Gene in vivo to Animal **Tissue**

Detail Description Paragraph:
[0175] In order to demonstrate that bactofection can occur in vivo, restrained **mice** (Balb/c) were intranasally inoculated with 5.times.10.sup.6 viable S. flexneri .DELTA.aro.DELTA.virG containing either p.beta.-gal+SV or p.beta.-gal-SV in a volume of 10 .mu.l of PBS. 48 hours after inoculation, the **mice** were sacrificed, **lung tissue** collected and frozen to -70.degree. C. Cryosections (5.0 .mu.M) were prepared, fixed, and then stained overnight for .beta.-gal activity as described above (Hawley-Nelson et al, supra). Following staining, the sections were rinsed twice with PBS, then sealed under coverslips.

Detail Description Paragraph:
[0176] Blue-staining .beta.-gal-positive cells were visible per **lung** section infected with p.beta.-gal+SV, but not those infected with .beta.-gal-SV.

Detail Description Paragraph:
[0182] In order to show another example of in vivo use of bactofection, 5.times.10.sup.7 S. flexneri .DELTA.aro .DELTA.virG containing the pCEP4::gp160 plasmid construct were administered intranasally to restrained Balb/c **mice**. 14 days following bactofection, the **mice** were sacrificed and **spleens** collected.

Detail Description Paragraph:
[0184] Splenocytes **isolated from mice** bactofected with plasmid pCEP4::gp160, containing the gene for HIV-1 gp160, showed a seven-fold stimulation, while splenocytes from control (pCEP4) bactofected **mice** showed no response.

CLAIMS:

6. The method of claim 1, wherein said invasive bacteria is selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

5. Document ID: US 20030035810 A1

L29: Entry 5 of 16 File: PGPB Feb 20, 2003

DOCUMENT-IDENTIFIER: US 20030035810 A1
TITLE: Microbial delivery system

Brief Description of Drawings Paragraph:
[0015] FIG. 3. Results of ELISA analysis of Ara h 2-specific IgG antibodies produced in **mice** following injection of E. coli producing Ara h 2. IgG1 is on the left and IgG2a is on the right.

Brief Description of Drawings Paragraph:

[0016] FIG. 4. Results of ELISA analysis of Ara h 3-specific IgG antibodies produced in **mice** following injection of E. coli producing Ara h 3. IgG1 is on the left and IgG2a is on the right.

Detail Description Paragraph:
[0026] **"Inducible promoter": The term "inducible promoter"**, as used herein, means a promoter site which is activated directly by the presence or absence of a chemical agent or indirectly by an environmental stimulus such as **temperature** changes. A promoter is the region of DNA at which the enzyme RNA polymerase binds and initiates the process of gene transcription.

Detail Description Paragraph:
[0051] Methods of inducing transcription include but are not limited to induction by the presence or absence of a chemical agent, **induction using a nutrient starvation inducible promoter, induction using a phosphate starvation inducible promoter and induction using a temperature sensitive inducible promoter**. A particularly preferred system for regulating gene expression utilizes tetracycline controllable expression system. Systems which utilize the tetracycline controllable expression system are commercially available (see for example, Clontech, Palo Alto, Calif.).

Detail Description Paragraph:
[0077] Adjuvants that are known to stimulate Th2 responses are preferably avoided. Particularly preferred adjuvants include, for example, preparations (including heat-killed samples, extracts, partially purified **isolates**, or any other preparation of a microorganism or macroorganism component sufficient to display adjuvant activity) of microorganisms such as Listeria monocytogenes or others (e.g., Bacille Calmette-Guerin [BCG], Corynebacterium species, Mycobacterium species, Rhodococcus species, Eubacteria species, Bortadella species, and Nocardia species), and preparations of nucleic acids that include unmethylated CpG motifs (see, for example, U.S. Pat. No. 5,830,877; and published PCT applications WO 96/02555, WO 98/18810, WO 98/16247, and WO 98/40100, each of which is incorporated herein by reference).

Detail Description Paragraph:
[0079] If adjuvants are not synthesized by microorganisms in accordance with the present invention, adjuvants which are cytokines may be provided as impure preparations (e.g., **isolates** of cells expressing a cytokine gene, either endogenous or exogenous to the cell), but are preferably provided in purified form. Purified preparations are preferably at least about 90% pure, more preferably at least about 95% pure, and most preferably at least about 99% pure. Alternatively, genes encoding the cytokines or immunological inducing agents may be provided, so that gene expression results in cytokine or immunological inducing agent production either in the individual being treated or in another expression system (e.g., an in vitro transcription/translation system or a host cell) from which expressed cytokine or immunological inducing agent can be obtained for administration to the individual. It is recognized that microorganisms utilized to synthesize and deliver allergenic and/or immunomodulatory proteins according to the present invention can act as an adjuvant, and that preferred microorganisms are immunostimulatory adjuvants.

Detail Description Paragraph:
[0087] In order to prolong the effect of an agent, it is often desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the agent then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of

the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of agent to polymer and the nature of the particular polymer employed, the rate of release of the agent can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides) Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body **tissues**.

Detail Description Paragraph:
[0100] Methods of encapsulating live cells are known and may also be used in accordance with the present invention for delivering antigen-secreting microorganisms to individuals. The following references are provided as examples of encapsulation of live cells. However, any method of encapsulating live cells may be used in the present invention. U.S. Pat. No. 5,084,350; U.S. Pat. No. 4,680,174; and U.S. Pat. No. 4,352,883 (all of which are incorporated herein by reference) describe the encapsulation of a prokaryotic or eukaryotic cell or cell culture in microcapsules. Briefly, U.S. Pat. No. 5,084,350; 4,680,174; and 4,352,883 disclose that a **tissue** sample, cell, or cell culture to be encapsulated is first prepared in finely divided form in accordance with well-known techniques and suspended in an aqueous medium suitable for maintenance and for supporting the ongoing metabolic processes of the particular cells involved. Media suitable for this purpose generally are available commercially. Thereafter, a water-soluble substance which is physiologically compatible with the cells and which can be rendered water-insoluble to form a shape-retaining coherent spheroidal mass or other shape is added to the medium. The solution is then formed into droplets containing cells together with their maintenance or growth medium and is immediately rendered water-insoluble and gelled to form shape-retaining, typically spheroidal coherent masses.

Detail Description Paragraph:
[0103] The compositions of the present invention may be employed to treat or prevent allergic reactions in a subject. Subjects are animal and human patients in need of treatment for allergies. Preferably, the animal is a domesticated mammal (e.g., a dog, a cat, a horse, a sheep, a pig, a goat, a cow, etc). Animals also include laboratory animals such as **mice**, rats, hamsters, monkeys, and rabbits. Any individual who suffers from allergy, or who is susceptible to allergy, may be treated. It will be appreciated that an individual can be considered susceptible to allergy without having suffered an allergic reaction to the particular antigen in question. For example, if the individual has suffered an allergic reaction to a related antigen (e.g., one from the same source or one for which shared allergies are common), that individual will be considered susceptible to allergy to the relevant antigen. Similarly, if members of an individual's family are allergic to a particular antigen, the individual may be considered to be susceptible to allergy to that antigen. More preferably, any individual who is susceptible to anaphylactic shock upon exposure to food allergens, venom allergens or rubber allergens may be treated according to the present invention.

Detail Description Paragraph:
[0109] The following experiments describe the encapsulation of allergens in bacteria for use as a delivery vehicle and/or adjuvant in immunotherapy in accordance with the teachings of the present invention. Recombinant peanut allergen proteins (Ara h 1, Ara h 2, and Ara h 3; Burks et al. J Allergy Clin Immunol. 88 (2):172-9, 1991; Burks et al. J Allergy Clin Immunol. 90(6 Pt 1):962-9, 1992; Rabjohn et al. J Clin Invest. 103(4):535-42, 1999; incorporated herein by reference) were produced in E. coli BL21 cells by transforming the bacterial cells with cDNA clones encoding the proteins (see Appendix B; sequences cloned into pET24, Novagen, Madison, Wis.). The transformed cells were then injected into C3H/HEJ **mice** to determine if the allergen-expressing E. coli elicited an immune response.

Detail Description Paragraph:

[0111] The following protocol was developed for the preparation of allergen-producing E. coli cells for inoculation of **mice**.

Detail Description Paragraph:
Immune Response of **Mice**

Detail Description Paragraph:
[0126] The following protocol was utilized to determine the immune response of **mice** injected with allergen-producing bacteria. Blood was collected from the tail vein of each **mouse** used before the first injection. Enough blood was collected for antibody ELISA for each allergen and E. coli proteins. On Day Zero each **mouse** was injected with 100 microliters of the killed E. coli samples subcutaneously in the left hind flank. The **mice** were injected for the second time on Day 14 using the same procedure as Day Zero. On Day 21, a second blood sample was collected from each **mouse**. Blood samples at Day 0 and Day 21 were assayed for IgG1 and IgG2a antibodies to either Ara h 1, Ara h 2, or Ara h 3 by an ELISA assay.

Detail Description Paragraph:
[0127] **Mice** injected with E. coli producing Ara h 1 did not give detectable levels of any immunoglobulin to the Ara h 1 allergen and therefore, that data are not shown. Without limitation to theory, we speculate that this may be due to the relatively small amounts of Ara h 1 produced by these cells (see previous discussion). **Mice** injected with E. coli producing Ara h 2 contained relatively high levels of IgG1 and IgG2a. Again, without limitation to the cause, we speculated that this may be due to the amount of Ara h 2 released from these cells (see discussion above). **Mice** injected with E. coli producing Ara h 3 contained relatively high levels of IgG2a (indicative of a Th1-type response) and elicited relatively low levels of IgG1 (indicative of a Th2-type response.

Detail Description Paragraph:
[0129] The present data should be cautiously interpreted. The data in the Figures only represent O.D. levels and do not represent absolute amounts of immunoglobulin. Therefore comparisons between groups should take into consideration the data presented as O.D. However, the general trend suggests that for example, more **mice** exhibited an IgG2a response to Ara h 3 than **mice** that exhibit an IgG1 response to Ara h 3.

CLAIMS:

4. The method of claim 1, wherein in the step of providing, the microorganism is selected from the group consisting of: E. coli, Lactococcus, **Listeria**, Vibrio, Salmonella and S. cerevisiae.

20. The composition of claim 16, wherein the microorganism is selected from the group consisting of: E. coli, Lactococcus, **Listeria**, Vibrio, Salmonella and S. cerevisiae

6. Document ID: US 20030027286 A1

L29: Entry 6 of 16 File: PGPB Feb 6, 2003

DOCUMENT-IDENTIFIER: US 20030027286 A1

TITLE: Bacterial promoters and methods of use

Summary of Invention Paragraph:
[0007] 1. An **isolated** nucleic acid comprising a fusion promoter said fusion promoter comprising at least one promoter that is modified to have altered activity in at least one gram-positive organism said promoter being linked to at least one operator selected from the group consisting of xylO, tetO, trpO, malO and .lambda.clO, wherein said at least one operator is positioned such that binding of at least one repressor to said at least one operator represses transcription from said fusion promoter.

Summary of Invention Paragraph:
[0008] 2. The **isolated** fusion promoter of claim 1, wherein said at least one promoter is selected from the group consisting of SEQ ID NOs.: 36-45.

Summary of Invention Paragraph:
[0009] 3. The **isolated** nucleic acid of claim 1 wherein said at least one operator is xylO.

Summary of Invention Paragraph:
[0010] 4. The **isolated** nucleic acid of claim 3, wherein said at least one promoter is T5.

Summary of Invention Paragraph:
[0011] 5. The **isolated** nucleic acid of claim 3, further comprising a second operator.

Summary of Invention Paragraph:
[0012] 6. The **isolated** nucleic acid of claim 5, wherein said second operator is lacO.

Summary of Invention Paragraph:
[0013] 7. The **isolated** nucleic acid of claim 1, wherein said fusion promoter is responsive to an inducer.

Summary of Invention Paragraph:
[0014] 8. The **isolated** nucleic acid of claim 7, wherein said inducer is xylose.

Summary of Invention Paragraph:
[0015] 9. The **isolated** nucleic acid of claim 1, wherein said fusion promoter is titratable.

Summary of Invention Paragraph:
[0016] 10. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

Summary of Invention Paragraph:
[0017] 11. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is Staphylococcus aureus.

Summary of Invention Paragraph:
[0018] 12. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is Enterococcus faecalis.

Summary of Invention Paragraph:
[0019] 13. An **isolated** nucleic acid comprising a fusion promoter said fusion promoter comprising at least one promoter selected from the group consisting of T5, CP25, P32, P59, P1P2 and PL, said promoter being linked to at least one operator selected from the group consisting of xylO, tetO, trpO, malO and .lambda.clO, wherein said at least one operator is positioned such that binding of at least one repressor to said at least one operator represses transcription from said fusion promoter.

Summary of Invention Paragraph:
[0020] 14. The **isolated** nucleic acid of claim 13, wherein said at least one operator is xylO.

Summary of Invention Paragraph:
[0021] 15. The **isolated** nucleic acid of claim 14, wherein said at least one promoter is T5.

Summary of Invention Paragraph:
[0022] 16. The **isolated** nucleic acid of claim 14, further comprising a second operator.

Summary of Invention Paragraph:
[0023] 17. The **isolated** nucleic acid of claim 16, wherein said second operator is lacO.

Summary of Invention Paragraph:
[0024] 18. The **isolated** nucleic acid of claim 13, wherein said fusion promoter is responsive to an inducer.

Summary of Invention Paragraph:
[0025] 19. The **isolated** nucleic acid of claim 18, wherein said inducer is xylose.

Summary of Invention Paragraph:
[0026] 20. The **isolated** nucleic acid of claim 13, wherein said fusion promoter is titratable.

Summary of Invention Paragraph:
[0027] 21. The **isolated** nucleic acid of claim 13, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

Summary of Invention Paragraph:
[0028] 22. The **isolated** nucleic acid of claim 13, wherein said at least one gram-positive organism is Staphylococcus aureus.

Summary of Invention Paragraph:
[0029] 23. The **isolated** nucleic acid of claim 13, wherein said at least one gram-positive organism is Enterococcus faecalis.

Summary of Invention Paragraph:
[0030] 24. An **isolated** fusion promoter comprising one of SEQ ID NO.: 26-35.

Summary of Invention Paragraph:
[0031] 25. A vector comprising the **isolated** nucleic acid of claim 1.

Summary of Invention Paragraph:
[0032] 26. A vector comprising the **isolated** nucleic acid of claim 13.

Summary of Invention Paragraph:
[0033] 27. A vector comprising the **isolated** nucleic acid of claim 24.

Summary of Invention Paragraph:
[0264] By "inhibit activity of a gene or gene product" is meant having the ability to interfere with the function of a gene or gene product in such a way as to decrease expression of the gene, in such a way as to reduce the level or activity of a product of the gene or in such a way as to inhibit the interaction of the gene or gene product with other biological molecules required for its activity. Agents which inhibit the activity of a gene include agents that inhibit transcription of the gene, agents that inhibit processing of the transcript of the gene, agents that reduce the **stability** of the transcript of the gene, and agents that inhibit translation of the mRNA transcribed from the gene. In microorganisms, agents which inhibit the activity of a gene can act to decrease expression of the operon in which the gene resides or alter the folding or processing of operon RNA so as to reduce the level or activity of the gene product. The gene product can be a non-translated RNA such as ribosomal RNA, a translated RNA (mRNA) or the protein product resulting from translation of the gene mRNA. Of particular utility to the present invention are antisense RNAs that have activities against the operons or genes to which they specifically hybridize.

Detail Description Paragraph:
[0333] Once the construct comprising the candidate nucleic acid operably linked to a promoter of the invention is made, it is transferred to host bacteria. Next, the proliferation of microorganisms transcribing a higher level of the candidate nucleic acid is compared to the proliferation of microorganisms that transcribe a lower level of the candidate nucleic acid or that do not transcribe the candidate nucleic acid. These varying levels of transcription can be achieved by varying the amounts of inducer (e.g., xylose, tetracycline, maltose, the absense of tryptophan, **temperature**, exposure to ultraviolet radiation, or analogs thereof). The level of maximal **induction that can be achieved will depend on the fusion promoter** selected. A difference in cell proliferation in the sample transcribing a higher level of the candidate nucleic acid compared to the sample expressing a lower level of the candidate nucleic acid indicates that the candidate nucleic acid encodes a nucleic acid that is complementary to an essential gene or encodes a peptide or protein that reduces proliferation of the microorganism.

Detail Description Paragraph:
[0340] In one embodiment, the present invention utilizes an antisense-based method to identify proliferation-required sequences. Generally, a library of nucleic acids from a given source are subcloned or otherwise inserted immediately downstream of an **inducible fusion promoter** on an appropriate vector, such as one of the expression vectors described herein, thus forming an expression library. It is generally preferred that expression is directed by a regulatable fusion promoter such that expression level can be adjusted by addition of variable concentrations of an inducer molecule or of an inhibitor molecule to the medium. **Temperature** activated fusion promoters, such as promoters regulated by **temperature** sensitive repressors, such as the lambda C.sub.1857 repressor, are also envisioned. Although the insert nucleic acids may be derived from the chromosome of the cell or microorganism into which the expression vector is to be introduced, because the insert is not in its natural chromosomal location, the insert nucleic acid is an exogenous nucleic acid for the purposes of the discussion herein.

Detail Description Paragraph:
[0342] Expression of the nucleic acid fragments in the test population of cells containing the expression library is then activated. Activation of the expression

vectors consists of subjecting the cells containing the vectors to conditions that result in the expression of the nucleic acids carried by the expression library. The test population of cells is then assayed to determine the effect of expressing the nucleic acid fragments on the test population of cells. Those expression vectors that negatively impact the growth of the cells upon induction of expression of the random sequences contained therein are identified, **isolated**, and purified for further study.

Detail Description Paragraph:
[0358] In some embodiments, promoter replacement and operator insertion methods are conducted in cells which have an increased frequency of homologous recombination. For example, the organism may lack or have a reduced level or activity of one or more exonucleases which would ordinarily degrade the DNA to be inserted into the chromosome, thereby effectively increasing the **stability** of the linear DNA in the organism.

Detail Description Paragraph:
[0392] Alternatively, in some embodiments, the operator insertion construct may be a single stranded nucleic acid, such as a synthetic oligonucleotide. Preferably, the single stranded nucleic acid is introduced into cells constitutively or conditionally expressing a protein which stabilizes single stranded DNA, such as the .lambda. Bet protein or an analogous protein, as described in Ellis et al., PNAS 98: 6742-6746, 2001, the disclosure of which is incorporated herein by reference in its entirety. After introduction of the operator insertion construct into a suitable cell, cells in which the operator has integrated by homologous recombination are identified. The cells may be identified by performing an amplification reaction or Southern blot. Alternatively, to identify cells in which expression of a gene required for proliferation is regulated by the inserted operator, cells which grow well under conditions in which the promoter which is regulated by the operator is active at a higher level but not under conditions in which the promoter is active at a lower level are identified. For example, cells which grow on media containing an **inducer which increases the level of transcription from the promoter** but not on media in which transcription is repressed may be identified. In some embodiments, the operator may be the lac operator and cells which grow on media containing IPTG but not on media lacking IPTG may be identified. In some embodiments, the operator may be the tet operator and cells which grow on media containing tetracycline but not on media lacking tetracycline may be identified. In some embodiments, the operator may be the xyl operator and cells which grow on media containing xylose but not on media lacking xylose may be identified. In some embodiments, the operator may be the mal operator and cells which grow on media containing maltose but not on media lacking maltose may be identified. In some embodiments, the operator may be the trp operator and cells which grow on media lacking tryptophan but not on media containing tryptophan may be identified. In some embodiments, the operator may be the .lambda.cl operator and cells which grow at the premissive **temperature** but not at the restrictive **temperature** may be identified.

Detail Description Paragraph:
[0406] Since the expression of essential genes is required for proliferation of the organism, when such integration events occur within or near an essential gene, induction of transcription will be lethal or will severely impair proliferation of the organism. Such conditional lethal or conditional growth clones can be readily **isolated** and the genes into which the vector has integrated can be determined by the cloning and sequencing techniques described herein. In this manner, the random promoter insertion method can be used to discover essential bacterial genes.

Detail Description Paragraph:
[0411] Following the subcloning of the antisense nucleic acids, which are complementary to proliferation-required sequences or portions thereof from the host organism from which the stabilized antisense nucleic acids were originally

obtained, into an expression vector under control of a regulatable fusion promoter capable of replicating and producing stabilized transcripts in a second cell or microorganism of interest (i.e. a cell or microorganism other than the one from which the identified nucleic acids were obtained), the antisense nucleic acids are conditionally transcribed to test for bacterial growth inhibition. The coding sequences complementary to the antisense nucleic acids from the original host that, when transcribed, inhibit growth of the second cell or microorganism are compared to the known genomic sequence of the second cell or microorganism to identify the homologous gene from the second organism. If the homologous sequence from the second cell or microorganism is not known, it may be identified and **isolated** by hybridization to the proliferation-required sequence of interest from the original host organism or by amplification using PCR primers based on the proliferation-required nucleotide sequence of interest. In this way, nucleotide sequences which may be required for the proliferation of the second cell or microorganism may be identified. For example, the second microorganism may be selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans or any species falling within the genera of any of the above species.

Detail Description Paragraph:
[0413] In another embodiment of the present invention, screening of candidate antibiotic compounds can be performed directly by using the antisense molecule **isolated** from the original host organism. In this embodiment, an antisense nucleic acid comprising a nucleic acid complementary to the proliferation-required sequences from the original host organism or a portion thereof is transcribed in the second host from a regulatable fusion promoter such as one of the fusion promoters described herein. If the antisense molecule is transcribed so as to sufficiently alter the level or activity of a nucleic acid required for proliferation of the second host, the second host may be used directly in a cell-based assay, such as those described herein, to identify candidate antibiotic compounds.

Detail Description Paragraph:
[0415] Other embodiments concern an in vivo system for determining whether a gene is required for infection of a host by a bacterium. Such a system can also be used for validating the drug discovery targets and lead compounds identified by the approaches described above. Accordingly, bacterial strains (e.g., including bacterial strains such as Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidernidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans or any species falling within the genera of any of the above species) having essential genes under the control of the regulatable promoters described herein are created using the EGI technology, promoter replacement technology, operator insertion technology or the random promoter insertion technology and these bacteria are used to inoculate a suitable host animal (e.g., neutropenic **mice**). Two hours after inoculation, an experimental group of animals are either provided a solution containing inducer (if the EGI technology or random promoter insertion technology was used to create the compromised cells) or a solution containing a minimal amount of inducer (if the promoter replacement technology or operator insertion technology was used to create the compromised cells). Accordingly, the bacteria are induced to generate the transcript or peptide that interacts with the essential gene or gene product or transcription of an endogenous essential gene is reduced. Subsequently, the amount of viable bacteria remaining in the animal or **tissue** sample from the animal is

determined. By using this approach, one can rapidly evaluate bacterial strains carrying essential genes that can be selectively downregulated in vivo. Additionally, one can use these animal models to screen lead compounds in vivo so as to identify new antibiotics.

Detail Description Paragraph:
[0421] To facilitate the construction of fusion promoters that function in Enterococcus faecalis, a reporter plasmid having a swapable promoter module located between a xyl repressor gene (xylR) and xyl operator (xylO) was designed. The Lactococcus lactis promoter probe vector pAK80 (described in Israelsen, et al. Appl. Environ. Microbiol. 61, 2540-47 (1995), the disclosure of which is incorporated by reference in its entirety), which contains replicons functional in both Escherichia coli (p15A origin) and Enterococcus faecalis (PCT 1138 replicon) as well as a selectable marker for erythromycin resistance (erm), was digested at the unique HindIII and BamHI polylinker sites just upstream from the promoterless Leuconostoc mesenteroides .beta.-galactosidase reporter genes (lacL-lacM). After digestion, the 11 kb vetor fragment was purified from the 26 bp polylinker by preparative gel electrophoresis then ligated to a 1535 bp gel purified HindIII/BamHI fragment from pEP25 (SEQ ID NO: 7) which contains the CP25 promoter from Lactococcus lactis (SEQ ID NO.: 38) flanked upstream by the xylose repressor gene (xylR) and downstream by the xylose operator sequence (xylO). A portion of the resulting ligation mixture was transformed into competent Escherichia coli XL-1 Blue cells (Stratagene, La Jolla, Calif.) and 20 .mu.l, 75 .mu.l or 100 .mu.l of the transformation mixture was plated on BYGT medium containing 20 .mu.g/ml erythromycin. **Isolated** erythromycin-resistant transformants were picked and streaked to obtain single colony **isolates**. Plasmid DNA was then purified from representative single colony transformants. The presence of the chimeric plasmid pEPEF1 (SEQ ID NO.: 8) was confirmed by digesting purified plasmid preparations with HindIII/BamHI and detecting an excised 1535 bp fragment, which corresponds to the fragment containing the Xyl-CP25 fusion promoter, by gel electrophoresis.

Detail Description Paragraph:
[0443] In another example, expression vectors functional in both Escherichia coli and Enterococcus faecalis were constructed by replacing the lacL-lacM reporter genes of pEPEF1 with the multiple cloning site (MCS) and rrnBtlt2 terminator of pLexP20. Specifically, pEPEF1 was digested with SmaI and SalI to remove the 3808 basepair fragment containing the lacL-lacM reporter genes. Following digestion, the large vector fragment was gel purified, the SalI overhanging end was made blunt with T4 DNA polymerase, and the fragment was circularized by ligating the blunt ends. The resulting plasmid, termed pEPEF2, was then digested with BamHI, gel purified, and blunted with T4 DNA polymerase. The ClaI/BstB1 fragment containing the MCS and rnnBtlt2 terminator was removed from pLexP20, gel purified, blunted using T4 DNA polymerase, then ligated to the blunt ended pEPEF2. A portion of the resulting ligation mixture was transformed into competent Escherichia coli and aliquots of the transformation mixture were plated on medium containing 20 .mu.g/ml erythromycin. **Isolated** erythromycin-resistant transformants were picked and streaked to obtain single colony **isolates**. Plasmid DNA was then purified from representative single colony transformants. The presence and orientation of the MCS/terminator fragment was determined by PCR and DNA seqeuncing, respectively. A plasmid comprising the MCS/terminator fragment oriented such that the MCS was located adjacent to xylO was termed pEPEF3 (SEQ ID NO.: 17).

Detail Description Paragraph:
[0488] Nucleic acids involved in proliferation of Staphylococcus aureus were identified using the EGI technique as follows. A shotgun library of Staphylococcus aureus genomic fragments was cloned into the vector pXyIT5-P15a, which harbors the Xyl-T5 inducible promoter. The vector was linearized at a unique BamHI site immediately downstream of the XyIT5 promoter/operator. The linearized vector was treated with shrimp alkaline phosphatase to prevent reclosure of the linearized ends. Genomic DNA **isolated** from Staphylococcus aureus strain RN450 was fully

digested with the restriction enzyme Sau3A or alternatively, partially digested with DNase I and "blunt-ended" by incubating with T4 DNA polymerase. Random genomic fragments between 200 and 800 base pairs in length were selected by gel purification. The size-selected genomic fragments were added to the linearized and dephosphorylated vector at a molar ratio of 0.1 to 1, and ligated to form a shotgun library.

Detail Description Paragraph:
[0492] Nucleic acids involved in proliferation of E. faecalis were identified as follows. A shotgun library of E. faecalis genomic fragments was cloned into either pEPEF3 or pEPEF14, which contain the CP25 or P59 promoter, respectively, regulated by the xyl operator/repressor. The vector was linearized at a unique SmaI site immediately downstream of the promoter/operator. The linearized vector was treated with alkaline phosphatase to prevent reclosure of the linearized ends. Genomic DNA **isolated** from E. faecalis strain OG1RF was partially digested with DNase I and "blunt-ended" by incubating with T4 DNA polymerase. Random genomic fragments between 200 and 800 base pairs in length were selected by gel purification. The size-selected genomic fragments were added to the linearized and dephosphorylated vector at a molar ratio of 2 to 1, and ligated to form a shotgun library.

Detail Description Paragraph:
[0498] Expression vectors were purified from Staphylococcus aureus or Enterococcus faecalis colonies identified as having reduced proliferation upon induction of the fusion promoter. The nucleic acids contained on the expression vectors under control of the fusion promoter were **isolated** for subsequent nucleotide sequence determination and further characterization.

Detail Description Paragraph:
[0513] Plasmids from transformant colonies that received a dilution plating score of "2" or greater were **isolated** to obtain the genomic DNA insert responsible for growth inhibition as follows. Staphylococcus aureus were grown in standard laboratory media (LB or TB with 15 ug/ml Chloramphenicol to select for the plasmid). Growth was carried out at 37.degree. C. overnight in culture tubes or 2 ml deep well microtiter plates. Lysis was performed as follows. Cultures (2-5 ml) were centrifuged and the cell pellets resuspended in 1.5 mg/ml solution of lysostaphin (20 .mu.l/ml of original culture) followed by addition of 250 .mu.l of resuspension buffer (Qiagen). Alternatively, cell pellets were resuspended directly in 250 .mu.l of resuspension buffer (Qiagen) to which 5-20 .mu.l of a 1 mg/ml lysostaphin solution were added. DNA was **isolated** using Qiagen miniprep kits or Wizard (Qiagen) miniprep kits according to the instructions provided by the manufacturer.

Detail Description Paragraph:
[0524] For E. faecalis, plasmids from transformant colonies that received a dilution plating score of "2" or greater were **isolated** to obtain the genomic DNA insert responsible for growth inhibition as follows. E. faecalis were grown in THB 10 .mu.g/ml Erm at 30.degree. C. overnight in 100 ul culture wells in microtiter plates. To amplify insert DNA 2 ul of culture were placed into 25 .mu.l Qiagen Hot Start PCR mix. PCR reactions were in 96 well microtiter plates. Primers to vector regions flanking the insert were used in the PCR reaction. PCR was carried out in a PE GenAmp with the following cycle times:

Detail Description Paragraph:
Comparison of **Isolated** Nucleic Acids to Known Sequences

Detail Description Paragraph:
[0582] The strategy described in this example seeks to determine if a targeted gene within an operon is required for cell proliferation by replacing the targeted gene in the chromosome with an in-frame deletion of the coding region of the targeted gene. Deletion inactivation of a chromosomal copy of a gene in Staphylococcus

aureus or Enterococcus faecalis can be accomplished by integrative gene replacement. The principles of this method were described in Xia, M., et al. 1999 Plasmid 42:144-149 and Hamilton, C. M., et al 1989. J. Bacteriol. 171: 4617-4622, the disclosures of which are incorporated herein by reference in their entireties. In this approach, a mutant allele of the targeted gene is constructed by way of an in-frame deletion and introduced into the chromosome using a suicide vector. This results in a tandem duplication comprising a deleted (null) allele and a wild type allele of the target gene. Cells in which the vector sequences have been deleted are **isolated** using a counter-selection technique. Removal of the vector sequence from the chromosomal insertion results in either restoration of the wild-type target sequence or replacement of the wild type sequence with the deletion (null) allele. E. faecalis genes can be disrupted using a suicide vector that contains an internal fragment to a gene of interest. With the appropriate selection this plasmid will homologously recombine into the chromosome (Nallapareddy, S. R., X. Qin, G. M. Weinstock, M. Hook, B. E. Murray. 2000. Infect. Immun. 68:5218-5224, the disclosure of which is incorporated herein by reference).

Detail Description Paragraph:
[0586] Once a tetR Staphylococcus aureus strain is **isolated** from the above technique and shown to include truncated and wild-type alleles of the targeted gene as described above, a second plasmid, pSA7592 (Xia, M., et al. 1999 Plasmid 42:144-149, the disclosure of which is incorporated herein by reference in its entirety) is introduced into the strain by electroporation. This gene includes an erythromycin resistance gene and a repC gene that is expressed at high levels. Expression of repC in these transformants is toxic due to interference of normal chromosomal replication at the integrated pT181 origin of replication. This counterselects for strains that have removed the vector sequence by homologous recombination, resulting in either of two outcomes: The counterselected cells either possess a wild-type allele of the targeted gene or a gene in which the wild-type allele has been replaced by the engineered in-frame deletion of the truncated allele.

Detail Description Paragraph:
[0596] Antibodies capable of specifically recognizing the protein of interest can be generated using synthetic peptides using methods well known in the art. See, Antibodies: A Laboratory Manual, (Harlow and Lane, Eds.) Cold Spring Harbor Laboratory (1988). For example, 15-mer peptides having a sequence encoded by the appropriate identified gene sequence of interest or portion thereof can be chemically synthesized. The synthetic peptides are injected into **mice** to generate antibodies to the polypeptide encoded by the identified nucleic acid sequence of interest or portion thereof. Alternatively, samples of the protein expressed from the expression vectors discussed above can be purified and subjected to amino acid sequencing analysis to confirm the identity of the recombinantly expressed protein and subsequently used to raise antibodies.

Detail Description Paragraph:
Production of an Antibody to an **Isolated** Staphylococcus aureus or Enterococcus faecalis Protein

Detail Description Paragraph:
[0600] Substantially pure protein or polypeptide is **isolated** from the transformed cells as described in Example 16. The concentration of protein in the final preparation is adjusted, for example, by concentration on a 10,000 molecular weight cut off AMICON filter device (Millipore, Bedford, Mass.), to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the protein can then be prepared as follows:

Detail Description Paragraph:
[0601] Monoclonal antibody to epitopes of any of the peptides identified and **isolated** as described can be prepared from murine hybridomas according to the

classical method of Kohler, G. and Milstein, C., Nature 256:495 (1975) or any of the well-known derivative methods thereof. Briefly, a **mouse** is repetitively inoculated with a few micrograms of the selected protein or peptides derived therefrom over a period of a few weeks. The **mouse** is then sacrificed, and the antibody-producing cells of the **spleen isolated**. The **spleen** cells are fused by means of polyethylene glycol with **mouse** myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully-fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as described by Engvall, E., "Enzyme immunoassay ELISA and EMIT," Meth. Enzymol. 70:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. et al. Basic Methods in Molecular Biology Elsevier, New York. Section 21-2.

Detail Description Paragraph:
[0608] For the thigh infection model, animals are rendered neutropenic chemically prior to the start of the experiment. By one approach, the inoculum will consist of 10.sup.5 to 10.sup.6 cells of a normally virulent Gram-positive organism expressing antisense to an essential gene such as that for gyrB under control of a fusion promoter described herein. These cells are injected to one thigh of a suitable animal (e.g., **mice**). Most microorganisms attain a logarithmic growth 2 hours after inoculation. Treated animals receive therapy from 2 hours post inoculation up to 24 hours. A typical efficacy study consists of 2 control groups and 5 treatment groups, each being treated with different doses of inducer. With 15 animals per group, 105 **mice** are used per study. The control sets are given intraperitoneal injections of saline, which will not **induce expression of the fusion promoter**. The experimental animals are given the inducer (e.g. xylose, tetracycline, IPTG, maltose, absence of tryptophan, or **temperature** change) in intraperitoneal injections to **induce expression of the antisense promoter**. Alternatively, the **promoter can be induced** by intravenous infusion of inducer at sub-toxic levels.

Detail Description Paragraph:
[0609] The end point used to follow the infection process is viable bacterial counts per thigh. The controls in which the antisense RNA is not induced will fail to impede growth of the Gram-positive organism and thus a logarithmic increase in viable bacteria will occur. The Gram-positive cells recovered from the site of infection should be viable until antisense expression is subsequently induced. This will demonstrate that the plasmid is still patent. However, in animals receiving the xylose injections, expression of the antisense RNA will occur, the essential gene or gene product will be compromised, and the Gram-positive cells infecting the **mice** will not multiply. Accordingly, fewer viable cells will be recovered from the site of infection in the experimental animals. The Gram-positive cells from the induced **mice** will be recovered, if still present, and assayed as above to determine if the promoter and gene are still present and functional.

Detail Description Paragraph:
[0611] Having **isolated** and expressed bacterial proteins shown to be required for bacterial proliferation, the present invention further contemplates the use of these expressed target proteins in assays to screen libraries of compounds for potential drug candidates. The generation of chemical libraries is well known in the art. For example, combinatorial chemistry can be used to generate a library of compounds to be screened in the assays described herein. A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building block" reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining amino acids in every possible combination to yield peptides of a given length. Millions of chemical compounds

theoretically can be synthesized through such combinatorial mixings of chemical building blocks. For example, one commentator observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds. (Gallop et al., 1994, "Applications of Combinatorial Technologies to Drug Discovery, Background and Peptide Combinatorial Libraries" Journal of Medicinal Chemistry, 37: 1233-1250). Other chemical libraries known to those in the art may also be used, including natural product libraries.

Detail Description Paragraph:
[0614] To illustrate the screening process, the target polypeptide and chemical compounds of the library are combined with one another and permitted to interact with one another. A labeled substrate is added to the incubation. The label on the substrate is such that a detectable signal is emitted from metabolized substrate molecules. The emission of this signal permits one to measure the effect of the combinatorial library compounds on the enzymatic activity of target enzymes by comparint it to the signal emitted in the absence of combinatorial library compounds. The characteristics of each library compound are encoded so that compounds demonstrating activity against the enzyme can be analyzed and features common to the various compounds identified can be **isolated** and combined into future iterations of libraries.

Detail Description Paragraph:
[0625] In one embodiment of the cell-based assays, an antisense nucleic acid that is complementary to a proliferation-required gene from a Gram-positive organism is used to inhibit the production of a proliferation-required protein. Expression vectors which contain an antisense nucleic acid complementary to identified genes required for proliferation operably linked to a fusion promoter of the present invention are used to limit the concentration of a proliferation-required protein without severely inhibiting growth. To achieve that goal, a growth inhibition dose curve of inducer is calculated by plotting various doses of inducer against the corresponding growth inhibition caused by the antisense expression. From this curve, various percentages of antisense induced growth inhibition, from 1 to 100% can be determined. If the fusion promoter contained in the expression vector contains a xylO linked to one of the promoters of SEQ ID NO.: 36-45, transcription in Staphylococcus aureus and Enterococcus faecalis will be regulatable by the xyl repressor and expression from the **promoter can be induced** with xylose. Similarly, IPTG, xylose, tetracycline, maltose, absence of trp and **temperature inducible promoters** may be used. For example, the highest concentration of the inducer that does not reduce the growth rate significantly can be estimated from the curve. Cellular proliferation can be monitored by growth medium turbidity via OD measurements. In another example, the concentration of inducer that reduces growth by 25% can be predicted from the curve. In still another example, a concentration of inducer that reduces growth by 50% can be calculated. Additional parameters such as colony forming units (cfu) can be used to measure cellular viability.

Detail Description Paragraph:
[0652] To provide a consistent source of cells to screen, frozen stocks of host bacteria containing the desired antisense construct are prepared using standard microbiological techniques. For example, a single clone of the microorganism can be **isolated** by streaking out a sample of the original stock onto an agar plate containing nutrients for cell growth and an antibiotic for which the antisense construct contains a gene which confers resistance. After overnight growth an **isolated** colony is picked from the plate with a sterile needle and transferred to an appropriate liquid growth media containing the antibiotic required for maintenance of the plasmid. The cells are incubated at 30.degree. C. to 37.degree. C. with vigorous shaking for 4 to 6 hours to yield a culture in exponential growth. Sterile glycerol is added to 15% (volume to volume) and 100 .mu.L to 500 .mu.L aliquots are distributed into sterile cryotubes, snap frozen in liquid nitrogen, and stored at -80.degree. C. for future assays.

Detail Description Paragraph:
[0654] A day prior to an assay, a stock vial is removed from the freezer, rapidly thawed (37.degree. C. water bath) and a loop of culture is streaked out on an agar plate containing nutrients for cell growth and an antibiotic to which the antisense construct confers resistance. After overnight growth at 37.degree. C., ten randomly chosen, **isolated** colonies are transferred from the plate (sterile inoculum loop) to a sterile tube containing 5 mL of appropriate medium containing the antibiotic to which the antisense vector confers resistance. After vigorous mixing to form a homogeneous cell suspension, the optical density of the suspension is measured at 600 nm (OD.sub.600) and if necessary an aliquot of the suspension is diluted into a second tube of 5 mL, sterile, appropriate medium plus antibiotic to achieve an OD.sub.600.ltoreq.0.02 absorbance units. The culture is then incubated at 37.degree. C. for 1-2 hrs with shaking until the OD.sub.600 reaches OD 0.2-0.3. At this point the cells are ready to be used in the assay.

CLAIMS:

1. An **isolated** nucleic acid comprising a fusion promoter said fusion promoter comprising at least one promoter that is modified to have altered activity in at least one gram-positive organism said promoter being linked to at least one operator selected from the group consisting of xylO, tetO, trpO, malO and .lambda.clO, wherein said at least one operator is positioned such that binding of at least one repressor to said at least one operator represses transcription from said fusion promoter.

2. The **isolated** fusion promoter of claim 1, wherein said at least one promoter is selected from the group consisting of SEQ ID NOs.: 36-45.

3. The **isolated** nucleic acid of claim 1, wherein said at least one operator is xylO.

4. The **isolated** nucleic acid of claim 3, wherein said at least one promoter is T5.

5. The **isolated** nucleic acid of claim 3, further comprising a second operator.

6. The **isolated** nucleic acid of claim 5, wherein said second operator is lacO.

7. The **isolated** nucleic acid of claim 1, wherein said fusion promoter is responsive to an inducer.

8. The **isolated** nucleic acid of claim 7, wherein said inducer is xylose.

9. The **isolated** nucleic acid of claim 1, wherein said fusion promoter is titratable.

10. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

11. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is Staphylococcus aureus.

12. The **isolated** nucleic acid of claim 1, wherein said at least one gram-positive organism is Enterococcus faecalis.

13. An isolated nucleic acid comprising a fusion promoter said fusion promoter comprising at least one promoter selected from the group consisting of T5, CP25, P32, P59, P1P2 and PL, said promoter being linked to at least one operator selected from the group consisting of xylO, tetO, trpO, malO and .lambda.clO, wherein said at least one operator is positioned such that binding of at least one repressor to said at least one operator represses transcription from said fusion promoter.

14. The isolated nucleic acid of claim 13, wherein said at least one operator is xylO.

15. The isolated nucleic acid of claim 14, wherein said at least one promoter is T5.

16. The isolated nucleic acid of claim 14, further comprising a second operator.

17. The isolated nucleic acid of claim 16, wherein said second operator is lacO.

18. The isolated nucleic acid of claim 13, wherein said fusion promoter is responsive to an inducer.

19. The isolated nucleic acid of claim 18, wherein said inducer is xylose.

20. The isolated nucleic acid of claim 13, wherein said fusion promoter is titratable.

21. The isolated nucleic acid of claim 13, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

22. The isolated nucleic acid of claim 13, wherein said at least one gram-positive organism is Staphylococcus aureus.

23. The isolated nucleic acid of claim 13, wherein said at least one gram-positive organism is Enterococcus faecalis.

24. An isolated fusion promoter comprising one of SEQ ID NO.: 26-35.

25. A vector comprising the isolated nucleic acid of claim 1.

26. A vector comprising the isolated nucleic acid of claim 13.

27. A vector comprising the isolated nucleic acid of claim 24.

39. The vector of claim 25, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

60. The method of claim 48, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum,

Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes,** Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

75. The method of claim 63, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes,** Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

86. The method of claim 78, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes,** Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

98. The method of claim 90, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes,** Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

109. The method of claim 101, wherein said at least one gram-positive organism is selected from the group consisting of Bacillus anthracis, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium teteni, Corynebacterium diptheriae, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, **Listeria monocytogenes,** Mycobacterium leprae, Mycobacterium tuberculosis, Nocardia asteroides, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosis, Streptococcus pneumoniae, Streptococcus mutans and any species falling within the genera of any of the above species.

7. Document ID: US 20020123145 A1

L29: Entry 7 of 16 File: PGPB Sep 5, 2002

DOCUMENT-IDENTIFIER: US 20020123145 A1
TITLE: Methods for the replacement, translocation and stacking of DNA in eukaryotic genomes

Summary of Invention Paragraph:
[0005] The underlying reasons for the high variability in transgene expression in plants are not completely understood, but at least four factors are involved in this phenomenon. (1) **Tissue** culture: Somaclonal variation has long been associated with **tissue** culture regenerated plants. Changes in chromosome structure and ploidy, DNA sequence, DNA modification, and transposon activity have all been reported in somaclonal variants (Peschke and Phillips, 1992 Advances in Genetics, 30:41-75; Kaeppler et al., 2000 Plant Mol. Biol., 43:179-88). (2) Integration site: Chromosomal structures such as telomeres or heterochromatin are known to affect the expression of nearby genes (Stavenhagen and Zakian, 1994 Genes and Dev., 8:1411-22; Howe et al., 1995 Genetics, 140:1033-45; Wallrath and Elgin, 1995 Genes and Dev. 9:1263-77). As a transgene integrates at random locations, chromosomal influences on transgene expression can be expected to differ among independent transformants (Meyer, 2000 Plant Mol. Biol., 43:221-34). (3) Transgene redundancy: Transformed plants often contain variable numbers of transgenes. Rarely is there a positive correlation between gene expression and copy number. On the contrary, many cases have linked extra full or partial transgene copies to postrancriptional and transcriptional gene silencing (Muskens et al., 2000 Plant Mol. Biol., 43:243-60; Matzke et al., 2000 Plant Mol. Biol., 43:401-15). (4) Genetic mutations: As expected for any genetic manipulations, there is always the possibility of acquiring point mutations, deletions or rearrangements (Battacharyya et al., 1994 Plant J., 6:957-68).

Detail Description Paragraph:
[0043] In one embodiment of the present invention, the donor polynucleotide includes a promoter operably linked to a gene of interest. "Promoter" refers to a region of DNA involved in binding the RNA polymerase to initiate transcription. An "**inducible promoter**" refers to a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, **temperature**, pH, transcription factors and chemicals. A DNA segment is "operably linked" when placed into a functional relationship with another DNA segment. For example, DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers, for example, need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof.

Detail Description Paragraph:
[0046] A promoter can be naturally associated with the gene of interest, or it can be a heterologous promoter that is obtained from a different gene, or from a different species. Where direct expression of a gene in all **tissues** of a transgenic plant or other organism is desired, one can use a "constitutive" promoter, which is generally active under most environmental conditions and states of development or cell differentiation. Suitable constitutive promoters for use in plants include, for example, the cauliflower mosaic virus (CaMV) 35S transcription initiation region and region VI promoters, the 1'- or 2'-promoter derived from T-DNA of Agrobacterium tumefaciens, and other promoters active in plant cells that are known to those of skill in the art. Other suitable promoters include the full-length transcript promoter from Figwort mosaic virus, actin promoters, histone promoters, tubulin promoters, the mannopine synthase promoter (MAS), various ubiquitin or polyubiquitin promoters derived from, inter alia, Arabidopsis (Sun and Callis, 1997 Plant J., 11(5):1017-1027), the mas, Mac or DoubleMac promoters (described in U.S. Pat. No. 5,106,739 and by Comai et al., 1990 Plant Mol. Biol. 15:373-381) and other transcription initiation regions from various plant genes known to those of skill in the art. Such genes include for example, ACT11 from Arabidopsis (Huang et al., 1996 Plant Mol. Biol., 33:125-139), Cat3 from Arabidopsis (GenBank No. U43147,

Zhong et al., 1996 Mol. Gen. Genet., 251:196-203), the gene encoding stearoyl-acyl carrier protein desaturase from Brassica napus (GenBank No. X74782, Solocombe et al., 1994 Plant Physiol., 104:1167-1176), GPc1 from maize (GenBank No. X15596, Martinez et al., 1989 J. Mol. Biol., 208:551-565), and Gpc2 from maize (GenBank No. U45855, Manjunath et al., 1997 Plant Mol. Biol., 33:97-112).

Detail Description Paragraph:
[0049] Alternatively, one can use a promoter that directs expression of a gene of interest in a specific **tissue** or is otherwise under more precise environmental or developmental control. Such **promoters are referred to here as "inducible" or "repressible" promoters**. Examples of environmental conditions that may effect transcription by **inducible promoters** include pathogen attack, anaerobic conditions, ethylene, elevated **temperature** or the presence of light. Promoters under developmental control include promoters that initiate transcription only in certain **tissues**, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an **inducible promoter** may become fully or partially constitutive in certain locations. **Inducible promoters** are often used to control expression of the recombinase gene, thus allowing one to control the timing of the recombination reaction.

Detail Description Paragraph:
[0050] The **tissue**-specific E8 promoter from tomato is particularly useful for directing gene expression so that a desired gene product is located in fruits. See, e.g., Lincoln et al., 1988 Proc. Nat'l. Acad. Sci. USA, 84: 2793-2797; Deikman et al., 1988 EMBO J., 7: 3315-3320; Deikinan et al., 1992 Plant Physiol., 100: 2013-2017. Other suitable promoters include those from genes encoding embryonic storage proteins. Additional organ-specific, **tissue**-specific and/or inducible foreign promoters are also known (see, e.g., references cited in Kuhlemeier et al., 1987 Ann. Rev. Plant Physiol., 38:221), including those 1,5-ribulose bisphosphate carboxylase small subunit genes of Arabidopsis thaliana (the "ssu" promoter), which are light-inducible and active only in photosynthetic **tissue**, anther-specific promoters (EP 344029), and seed-specific promoters of, for example, Arabidopsis thaliana (Krebbers et al., 1988 Plant Physiol., 87:859). Exemplary green **tissue**-specific promoters include the maize phosphoenol pyruvate carboxylase (PEPC) promoter, small submit ribulose bis-carboxylase promoters (ssRUBISCO) and the chlorophyll a/b binding protein promoters. The promoter may also be a pith-specific promoter, such as the promoter **isolated** from a plant TrpA gene as described in International Publication No. WO/93/07278.

Detail Description Paragraph:
[0066] The construction of polynucleotide constructs generally requires the use of vectors able to replicate in bacteria. A plethora of kits are commercially available for the purification of plasmids from bacteria. For their proper use, follow the manufacturer's instructions (see, for example, EasyPrepJ, FlexiPrepJ, both from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The **isolated** and purified plasmids can then be further manipulated to produce other plasmids, used to transfect cells or incorporated into Agrobacterium tumefaciens to infect and transform plants. Where Agrobacterium is the means of transformation, shuttle vectors are constructed. Cloning in Streptomyces or Bacillus is also possible.

Detail Description Paragraph:
[0074] The polynucleotide constructs that include recombination sites and/or recombinase-encoding genes can be introduced into the target cells and/or organisms by any of the several means known to those of skill in the art. For instance, the DNA constructs can be introduced into plant cells, either in culture or in the organs of a plant by a variety of conventional techniques. For example, the DNA constructs can be introduced directly to plant cells using biolistic methods, such as DNA particle bombardment, or the DNA construct can be introduced using techniques such as electroporation and microinjection of plant cell protoplasts.

Particle-mediated transformation techniques (also known as "biolistics") are described in Klein et al., 1987 Nature, 327:70-73; Vasil, V. et al., 1993 Bio/Technol., 11:1553-1558; and Becker, D. et al., 1994 Plant J., 5:299-307. These methods involve penetration of cells by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface. The biolistic PDS-1000 Gene Gun (Biorad, Hercules, Calif.) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells. The process is applicable to a wide range of **tissues** and cells from organisms, including plants, bacteria, fungi, algae, intact animal **tissues**, **tissue** culture cells, and animal embryos. One can employ electronic pulse delivery, which is essentially a mild electroporation format for live **tissues** in animals and patients. Zhao, 1995 Advanced Drug Delivery Reviews, 17:257-262.

Detail Description Paragraph:
[0079] Transformed plant cells, derived by any of the above transformation techniques, can be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a **tissue** culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts **Isolation** and Culture, Handbook of Plant Cell Culture, pp. 124-176, Macmillian Publishing Company, New York (1983); and in Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, (1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar et al., J. **Tissue** Cult. Meth., 12:145 (1989); McGranahan et al., Plant Cell Rep., 8:512 (1990)), organs, or parts thereof. Such regeneration techniques are described generally in Klee et al., Ann. Rev. of Plant Phys., 38:467-486 (1987).

Detail Description Paragraph:
[0080] The methods are also useful for producing transgenic and chimeric animals of most vertebrate species. Such species include, but are not limited to, nonhuman mammals, including rodents such as **mice** and rats, rabbits, ovines such as sheep and goats, porcines such as pigs, and bovines such as cattle and buffalo. Methods of obtaining transgenic animals are described in, for example, Puhler, A., Ed., Genetic Engineering of Animals, VCH Publ., 1993; Murphy and Carter, Eds., Transgenesis Techniques: Principles and Protocols (Methods in Molecular Biology, Vol. 18), 1993; and Pinkert, Calif., Ed., Transgenic Animal Technology: A Laboratory Handbook, Academic Press, 1994. Transgenic fish having specific genetic modifications can also be made using the claimed methods. See, e.g., Iyengar et al., (1996) Transgenic Res. 5: 147-166 for general methods of making transgenic fish.

Detail Description Paragraph:
[0081] One method of obtaining a transgenic or chimeric animal having specific modifications in its genome is to contact fertilized oocytes with a vector that includes the polynucleotide of interest flanked by recombination sites. For some animals such as **mice**, fertilization is performed in vivo and fertilized ova are surgically removed. In other animals, particularly bovines, it is preferably to remove ova from live or slaughterhouse animals and fertilize the ova in vitro. See DeBoer et al., WO 91/08216. In vitro fertilization permits the modifications to be introduced into substantially synchronous cells. Fertilized oocytes are then cultured in vitro until a pre-implantation embryo is obtained containing about 16-150 cells. The 16-32 cell stage of an embryo is described as a morula. Pre-implantation embryos containing more than 32 cells are termed blastocysts. These embryos show the development of a blastocoel cavity, typically at the 64 cell stage. If desired, the presence of a desired exogenous polynucleotide in the embryo cells can be detected by methods known to those of skill in the art. Methods for culturing fertilized oocytes to the pre-implantation stage are described by Gordon et al., (1984) Methods Enzymol. 101: 414; Hogan et al., Manipulation of the **Mouse**

Embryo: A Laboratory Manual, C.S.H.L. N.Y. (1986) (**mouse** embryo); Hammer et al., (1985) Nature 315: 680 (rabbit and porcine embryos); Gandolfi et al., (1987) J. Reprod. Fert. 81: 23-28; Rexroad et al., (1988) J. Anim. Sci. 66: 947-953 (ovine embryos) and Eyestone et al., (1989) J. Reprod. Fert. 85: 715-720; Camous et al., (1984) J. Reprod. Fert. 72: 779-785; and Heyman et al., (1987) Theriogenology 27: 5968 (bovine embryos). Sometimes pre-implantation embryos are stored frozen for a period pending implantation. Pre-implantation embryos are transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is integrated. Chimeric mammals can be bred to form true germline transgenic animals.

Detail Description Paragraph:
[0093] The 84 bp .phi.C31 attP site (abbreviated as PP'), **isolated** as an ApaI-SacI fragment from pHS282 (Thorpe & Smith (1998) Proc. Nat'l. Acad. Sci. USA 95:5505-5510) was cloned into the same sites of the S. pombe integrating vector pJK148 (Keeney & Boeke (1994) Genetics 136:849-856) to make pLT44. This plasmid was targeted to the S. pombe leu1-32 allele by lithium acetate mediated transformation with NdeI cut DNA. The recipient host FY527 (h- ade6-M216 his3-D1 leu1-32 ura4-D18), converted to Leu+ by homologous recombination with pLT44, was examined by Southern analysis. One Leu+ transformant, designated FY527attP (FIG. 3A), was found to contain a single copy of pLT44. Another transformant, designated FY527attP.times.2 (FIG. 3B), harbors a tandem insertion of pLT44, and therefore contains two attP sites.

Detail Description Paragraph:
[0095] The S. pombe ura4.sup.+ gene, excised from pTZura4 (S. Forsburg) on a 1.8 kb EcoRI-BamHI fragment, was inserted into pJK148 cut with the same enzymes to create pLT40. The .phi.C31 attB site (abbreviated as BB'), **isolated** from pHS21 as a 500 bp BamHI-XbaI fragment, was ligated into pLT40 cut with those enzymes, creating pLT42. Most of the leu1 gene was removed from pLT42 by deleting a XhoI fragment to create pLT45. This left 229 bp of leu1 in pLT45 and reduced its transformation efficiency to that of a plasmid without any leu1 homology. pLT50, which has a second attB site in the same orientation immediately on the other side of ura4, was constructed by first subcloning the attB BamHI-SacI fragment from pLT42 into pUC19, excising it with EcoRI and SalII, and subsequently inserting it into pLT45 cut with EcoRI and XhoI. The second attB site in the final construct was sequenced once on each strand and found to be identical to the first attB site.

Detail Description Paragraph:
[0104] This experiment demonstrates that the .phi.C31 site-specific recombination system is an efficient means to deliver linear cDNAs into a target cell. To prepare cDNA substrates, the linear molecules would be linked by ligation or PCR synthesis attachment sites on both ends, followed by recombination with a tandem pair of chromosomally situated target sites, and replacement of the target DNA with the inserting cDNA. To test whether such a gene replacement reaction is efficient, an FY527 derivative bearing a tandem insertion of pLT44 was **isolated**. This strain, designated FY527attP.times.2, has two attP sites in direct orientation at the leu1 locus, separated by a leu1 gene and vector sequences (FIG. 2B). FY527attP.times.2 was transformed with linear DNA containing ura4.sup.+ flanked by attB sites. The linear substrate was obtained either as a gel-purified fragment from pLT50 (FIG. 2A) or as a PCR product from amplification of this plasmid. The plasmid pLT50, derived from pLT45, has a second directly oriented attB site on the other side of the ura4.sup.+ gene. Both linear substrates gave approximately the same transformation efficiency when co-transformed with pLT43, which stimulated the number of Ura+ transformants (Table 1). In some experiments, the frequency was as high as that of the replicating plasmid control.

Detail Description Paragraph:
[0109] In addition to these three classes of integration structures, there exists the possibility of integration patterns resulting from incomplete recombination of

attB.times.attP sites. This could occur if the amount of integrase protein is limiting, as it could be if pLT43 were lost from the cell. If the His.sup.+ phenotype is not selected for, His- colonies are readily found. Four possible structures could arise from a single recombination event between the four sites: 5'attB.times.5'attP, 3'attB.times.3'attB, 3'attB.times.5'attP and 5'attB.times.3'attP. If followed by a second attB.times.attP reaction, the 5'attB.times.5'attP and the 3'attB.times.3'attB integrants would be converted to the class 1 structure, and the 3'attB.times.5'attP and 5'attB.times.3'attP integrants would not be found, as the ura4.sup.+ marker would be deleted. One of the eight **isolates** gave a pattern consistent with the incorporation of intact pLT50 through a 5'attB.times.5'attP reaction. This class 4 structure is shown in FIG. 1G. The ura4 probe detected a single 2.3 kb band, and the leu1 probe detected bands of 3 kb, 5.6 kb and 18 kb. Cleavage with NdeI gave a 12 kb band that hybridized to both the leu1 and ura4 probes, consistent with physical linkage of the two markers. The remaining **isolate** had also incorporated the entire plasmid but had gained additional bands hybridizing to both leu1 and ura4. This represents a more complex event, perhaps indicating gene amplification at the locus.

Detail Description Paragraph:
[0138] Functional expression of the tk gene was tested with gancyclovir (Sigma Co.) treatment. The cells were seeded in 24-well **tissue** culture plate (1.times.10.sup.3 cells/well) and grown overnight. Gancyclovir (ranging from 0 to 50 mM) was added to each well, and cell growth was observed for several days. Wild type 293T cells were insensitive to gancyclovir up to the highest concentration tested (50 mM), whereas the two cell lines with the single Pc-PP'-tk-P'P fragment were sensitive to gancyclovir.

Detail Description Paragraph:
[0140] Four .mu.g of both pJHK1 and pJHK2 were co-transfected into 1.times.10.sup.6 293T cells that harbor a single copy of pJHK3. Three days after transfection, the cells were serially diluted and transferred to fresh DMEM containing 50 mM of hygromycin (Boehringer Mannheim) or gancyclovir. The resistant cells were **isolated** around 14 days after transfection, and further analyzed. For the transfection with linear DNA, the BB'-hpt-B'B linear fragment was prepared as a KpnI fragment purified from pJHK2.

Detail Description Paragraph:
[0142] Genomic DNA was **isolated** from 293T cells using QIAampR DNA Blood Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's manual. Genomic Southern hybridization was performed with standard protocol where DNA probe was made using random primed DNA labeling kit (Cat# 1004760) from Boehringer Mannheim.

Detail Description Paragraph:
[0219] A third test was Southern analysis of F1 floral and leaf **tissues**. DNA was cleaved with a combination of EcoRI, HindIII and SacI (FIG. 12A, 12B, depicted as E, H, S, respectively) and hybridized to a 35S probe. FIGS. 12A and 12B show the cleavage patterns expected from the parental and recombinant chromosomes. In CD426, the hybridization probe is expected to detect a single 3.1 kb band. In CD414, the probe should hybridize to two bands, one of a predict size of 2.5 kb, and the other a transgene-host border band of a size that depends on the position of the nearest host cleavage site. In a double recombination event that translocated the designated DNA fragment, the receptor chromosome should show two new bands of 2.2 and 1.1 while in the donor chromosome a single new 1.9 kb band and the same size transgene-host border fragment.

Detail Description Paragraph:
[0220] In instances where recombination was detected, the F1 plants were chimeric for the recombination event. The majority of the hybridization signal was to the parental fragments of 3.1 and 2.5 kb. However, when the blots were subjected to longer exposure times, a recombinant band was detected. Since intense hybridization

is seen in the .about.2 to 3.1 kb region, the expected 2.2 and 1.9 kb recombinant bands could not be observed over the background. However, the 1.1 kb band was clearly detected in some of the plants, in both floral and leaf **tissues**. This hybridization pattern was similar for F1 progenies from some other crosses. Both Southern and PCR data indicated that recombination took place in only a minor fraction of the cells.

CLAIMS:

15. The method of claim 14, wherein the irreversible recombinase polypeptide is a .phi.C31 integrase, a coliphage P4 recombinase, a coliphage lambda recombinase, a **Listeria** U153 or A118 phage recombinase, or an actinophage R4 Sre recombinase.

33. The method of claim 32, wherein the irreversible recombinase polypeptide is a .phi.C31 integrase, a coliphage P4 recombinase, a coliphage lambda recombinase, a **Listeria** U153 or A118 phage recombinase, or an actinophage R4 Sre recombinase.

49. The method of claim 40, wherein the irreversible recombinase polypeptide is a .phi.C31 integrase, a coliphage P4 recombinase, a coliphage lambda recombinase, a **Listeria** U153 or A118 phage recombinase, or an actinophage R4 Sre recombinase.

60. The method of claim 59, wherein the irreversible recombinase polypeptide is a .phi.C31 integrase, a coliphage P4 recombinase, a coliphage lambda recombinase, a **Listeria** U153 or A118 phage recombinase, or an actinophage R4 Sre recombinase.

8. Document ID: US 20020086837 A1

L29: Entry 8 of 16 File: PGPB Jul 4, 2002

DOCUMENT-IDENTIFIER: US 20020086837 A1
TITLE: Acne vaccine

Summary of Invention Paragraph:
[0002] It has been demonstrated that chemical agents including antibiotics, which reduce the numbers of this organism are therapeutic. In fact, it has been shown in patients failing to respond to erythromycin antibiotic therapy that P. acnes bacterium **isolated** from lesions of the skin acquired antibiotic resistance. Another agent benzoyl peroxide, the active ingredient in many of the over-the-counter acne products, has also been directly correlated with its effects on P. acnes colonization in the skin. Thus, it appears that in inflammatory acne, interference with colonization of skin follicles by the P. acnes bacterial species can prevent the most common form of inflammatory acne (whiteheads). This process of bacterial colonization of the skin follicle may provide a target for the acne gene therapy and vaccine.

Summary of Invention Paragraph:
[0009] Accordingly, it is an object of the invention to develop new approaches and vaccines for gene therapy of diseases. It is particularly an object of the invention to develop new therapies that are useful in treating acne and other pathological conditions. It is specifically an object to explore the use of a series of recombinant adenoviral vectors which contain genes encoding various

target antigens and/or immunostimulatory proteins to introduce into target **tissues** nucleic acids which can be used to treat diseased conditions in the host.

Summary of Invention Paragraph:
[0010] A specific aspect of the subject invention pertains to recombinant adenovirus vectors and/or recombinant viral particles that include, and support the replication and expression of, nucleic acids that encode both targeted pathogen antigens and, optionally, immunostimulatory molecules such as cytokines and co-receptors that enhance immunostimulation and/or chemotactic factors which recruit lymphocytes. These vectors can be administrated whereby expression of the cytokine and co-stimulatory molecule stimulate an immune response against the targeted antigens. Where the adenovirus expresses a chemokine as well, this vector will produce chemotactic factors within the injection site and would recruit T cells from the circulation into the **tissue** and initiate immunity. T cells stimulated in either scenario will react against the bacterial antigen resulting in destruction of bacteria.

Summary of Invention Paragraph:
[0011] Adenoviruses (Ads) have several properties that make them attractive for gene therapy. They can be grown to high titers. Human Ads can infect a variety of cell types, from a variety of species. In particular, adenovirus type 5 (Ad5) naturally infects human cells and can be used as a vector for the delivery of foreign genes to human **tissues**. Transduced genes can be expressed in non-dividing cells. A deletion of Ad early region 1 ("E1") can be combined with one in Ad early region 3 ("E3") (whose products are unnecessary for growth in culture); together these deletions increase the packaging capacity of the modified Ad to accommodate up to 8 kb of foreign DNA. Several non-defective and replication defective (E1-) have been characterized (see, e.g., Verna and Somia, Nature 389; 239-242 (1997); the E1 vectors are able to replicate only in certain cell lines such as the 293 cell line. El Ads can persist and continue to express in cultured cells and in vivo for extended periods. Since the virus does not integrate efficiently, expression should be transient, a possible advantage for certain gene therapy applications, particularly immunotherapy of bacterial infection.

Summary of Invention Paragraph:
[0014] The subject vaccination system provides several advantages over all other gene therapy approaches developed to date. Those skilled in the art will appreciate that commercially available gene therapy vectors (e.g., recombinant retrovirus, adenovirus associated virus; AAV, and DNA itself) can be used in accord with the principles of the subject invention. However, the adenovirus vector is a preferred vector, as it has several advantages. First, an ad vector is highly efficient in transferring genetic material (DNA) to the target cell. The virus genome (its genetic material which encodes the virus proteins) contains DNA and must enter the nucleus of infected cells in order to replicate. Thus, the structural molecules of the virus have evolved to facilitate the most efficient delivery for the viral DNA to the nucleus. Since in the recombinant virus we have cloned the gene we want encoded and expressed into the viral genome, this process of transfer can be matched by no other vector. Second, ad vectors have the ability to carry large segments of DNA (genetic information) up to 30,000 base pairs can be carried by a recombinant vector. Our typical target bacterial antigen is around 1000 base pairs which is similar in size to that of a cytokine molecule. Thus, a single vaccine could accommodate as many as 30 different bacterial target antigens or immune modifying cytokines, or any combination of both. Third, ad vectors have the ability to infect non-dividing cells. Other gene therapy vectors can only target dividing cells and would render them useless for targeting to muscle **tissue** as muscle cells are not an actively dividing cell type. Fourth, ad vector gene expression is transient in the target cell due to the lack of integration of the viral DNA into the host cell DNA. This is a highly desirable attribute of adenovirus vectors since longterm expression of immunomodulating molecules can be harmful. Other vectors are integrated into chromosomes and could cause insertional inactivation or mutation of

genes in such treated individuals.

Summary of Invention Paragraph:
[0016] Futher, the efficacy of the subject vaccination system can be bolstered by co-expression or co-administration of cytokines serving as powerful adjuvants in combination with the bacterial antigen targeted vector. Studies have shown that a class of soluble protein hormones called cytokines possess anti-tumor and anti-metastatic activity due to their ability to activate immune cells, such as T cells, which can recognize foreign elements unique to the tumor, known as antigens. T cells are capable of selectively attacking tumor tissue, leaving the normal tissue relatively unharmed. They can circulate throughout the body where they will identify tumor cells which have disseminated from the primary tumor site and destroy them. These same responses can be targeted toward attack of pathogenic bacteria. Cytokines are normally produced during the effector phases of natural and specific immunity. They mediate and regulate immune and inflammatory processes by stimulating cells that participate in an immune response to migrate and accumulate in inflamed tissues, by activating cellular functions that mediate the immune response, and by causing immune system cells to liberate signalling and effector substances. However, cytokines alone may not be sufficient for full activation of T cell responses which underly the specific component of adaptive immunity. Antigen presenting cells, those cells which are required to initiate T cell responses, express surface receptors, referred to as co-stimulatory molecules, which synergize with cytokines in the activation of T cells. For example, the cytokines interleukin-2 (IL-2) and interleukin-12 (IL-12) can promote anti-tumor activity through their ability to stimulate the cytolytic activity of T-cells, LAK cells (lymphokine activated killer cells), and TILS (tumor infiltrating lymphocytes). IL-2 is produced primarily by activated T-lymphocytes and by natural killer cells (NK) or LAK cells and acts in an autocrine and paracrine fashion to augment an immune response. It exerts regulatory effects on almost all cell types involved in immune responses; it stimulates the proliferation and differentiation of B-cells, T-cells, NK cells, LAK cells as well as the activation of monocytes and macro phages. It can also stimulate the production of other cytokines such IFN-g and TNF-a (Anderson, T. D., 1992, in: Cytokines in Health and Disease (eds. S. L. Kunkel and D. G. Remick); Marcel Dekker Inc., New York, pp.27-60). IL-12 on the other hand is secreted by professional antigen presenting cells (APCs) and serves to direct the development of immature T cells towards a T1-type cytokine profile which is characterized by the secretion of IFN-g and IL-2. IL-12 is a very potent inducer of IFN-g which accounts in part for its anti-tumor properties (Brunda, M. J. et al., 1995, J. Immunother. 17: 71). We have used the adenovirus expressing IL 12 to protect mice against L. major parasitic infection. Raja Gabaglia et al., J Immunol 162:753-760 (1999). This IL-12 treatment prevented the intracellular infection of macrophages by parasites and is a proof in principle that this vector will be a suitable adjuvant to prevent P. acnes colonization.

Summary of Invention Paragraph:
[0017] The mature form of IL-2 is a 133 amino acid secreted protein which ranges in molecular weight from 14 to 18 kD due to differential glycosylation. Human IL-2 is biologically active in a wide variety of species, and thus testing of its anti-tumor properties in various animal models is possible. IL-12 is a more complex protein consisting of two separate subunits of 35 and 40 kD in size. Both subunits are heavily glycosylated and they are linked in a 1:1 ratio by disulfide bonds. The murine cytokine is active in many species and thus it too can be used in a variety of animal models.

Summary of Invention Paragraph:
[0018] In order to be an effective anti-cancer treatment, high levels of cytokine are required at the site of the tumor. However, when delivered through the systemic circulation, high levels of cytokines such as IL-2 or IL-12 can result in immunotoxicities. To avoid elevated concentrations of circulating cytokines, we have developed adenovirus vectors which express either IL-2 or IL-12 and we have

shown them to be highly useful in the treatment of murine breast carcinoma. (Addison, C.L.et al, 1995, Proc. Natl. Acad. Sci. USA 92: 8522; Bramson et al., 1996, Hum. Gene Ther. 7:1995). By injecting the adenovirus directly into the **tumor** nodule, we are able to induce very high levels of cytokine expression with very little secretion into the serum (Bramson et al., 1996, Hum. Gene Ther. 7:1995). However, these treatments only lead to cures in 30-40% of the animals following a single inoculation. One way to improve the outcome of the therapy is to increase the immunogenicity of the **tumor**. In that way the **tumor** becomes a "better target" for the immune cells which are activated by the adenovirally delivered cytokine.

Summary of Invention Paragraph:
[0019] In the course of antigen presentation to a T cell, a number of sequential contacts need to be made between the T cell and the antigen presenting cell (APC) in order to ensure full activation and function of the T cell. These interactions occur between the major histocompatibility molecule-antigen complexes on the APC and the T cell receptor on the T cell. However, simply ligating the T cell receptor is insufficient for proper activation. The T cell must also ligate other molecules on the APC known as co-stimulatory molecules, including the B7-family of proteins and CD40. In the absence of co-stimulation, the T cell enters a state of non-responsiveness known as anergy. It has been clearly demonstrated in multiple models that the immunogenicity of **tumors** can be enhanced by the expression ofB7-1. Similarly another B7 family member, B7-2, can also improve **tumor** rejection. Thus, in the subject system, the addition of a co-stimulatory molecule such as B7-1 should improve anti-bacteria responses.

Detail Description Paragraph:
[0024] By "capable of facilitating an immune response", it is meant that a molecule (s) stimulates a cell(s) that participate in an immune response to migrate into and accumulate at **tissues** in which the molecule is present, and/or that said molecule (s) is (are) capable of stimulating cells of the immune system to engage in activities such as phagocytosis and cytolysis that are part of an immune response, and/or that said molecule(s) cause(s) immune system cells to liberate signalling and effector substances such as, for example, cytokines, antibodies and histamines.

Detail Description Paragraph:
[0026] Vectors, optionally containing a foreign nucleic acid, may be "introduced" into a host cell, **tissue** or organism in accordance with known techniques such as transformation, transfection using calcium-phosophate precipitated DNA, electroporation, particle bombardment, transfection with a recombinant virus or phagemid, infection with an infective viral particle, injection into **tissues** or microinjection of the DNA into cells or the like. Both prokaryotic and eukaryotic hosts may be employed, which may include bacteria, yeast, plants and animals, including human cells.

Detail Description Paragraph:
[0027] Once a given structural gene, cDNA or open reading frame has been introduced into the appropriate host, the host may be grown to express said structural gene, cDNA or open reading frame. Where the exogenous nucleic acid is to be expressed in a host which does not recognize the nucleic acid's naturally occurring transcriptional and translational regulatory regions, a variety of transcriptional regulatory regions may be inserted upstream or downstream from the coding region, some of which are externally inducible. Illustrative transcriptional regulatory regions or promoters for use in bacteria include the p-gal promoter, lambda left and right promoters, trp and lac promoters, trp-lac fusion promoter, and also the bacteriophage lambda Q operator and the C1857 **temperature**-sensitive repressor, for example, to provide for **temperature** sensitive expression of a structural gene. Regulation of the promoter is achieved through interaction between the repressor and the operator. For use in yeast, illustrative transcriptional regulatory regions or promoters include glycolytic enzyme promoters, such as ADH-I and -II promoters,

OPK promoter, and POI promoter, TRP promoter, etc.; for use in mammalian cells, transcriptional control elements include SV 40 early and late promoters, adenovirus major late promoter, etc. Other regulatory sequences useful in eukaryotic cells can include, for example, the cytomegalovirus enhancer sequence, which can be fused to a promoter sequence such as the SV40 promoter to form a chimeric promoter, or can be inserted elsewhere in the expression vehicle, preferably in close proximity to the promoter sequence. Where the **promoter is inducible**, permissive conditions may be employed (for example, **temperature** change, exhaustion, or excess of a metabolic product or nutrient, or the like).

Detail Description Paragraph:
[0031] It is important to recognize that the present invention is not limited to the use of such cells specifically exemplified herein. Cells from different species (human, **mouse**, etc.) or different **tissues** (breast epithelium, colon, neuronal **tissue**, lymphocytes, etc . . .) may also be used.

Detail Description Paragraph:
[0032] The term "pathogen" as used herein refers to viruses, bacteria, fungi, protozoa, parasites, or other microbes, organisms and agents that infect cell(s) and **tissues** thereby causing disease or other adverse symptoms. As particularly used herein, the term "pathogen" preferably refers to agents that have the capability to infect, or avoid destruction by, macrophages. Examples of such agents include, but are not limited to, P. acnes, L. monocytogenes, S. typhimurium, N. gonorrhoea, M avium, M. tuberculosis, M leprae, B. abortus, and C. albicans; and L. major,

Detail Description Paragraph:
[0034] "Fragment" or "subfragment" refers to an **isolated** nuclecic acid derived from a reference sequence by excising or deleting one or more nucleotides at any position of the reference sequence using known recombinant techniques, or by inserting a predetermined sequence of nucleotides at any predetermined position within the reference sequence using known recombinant techniques.

Detail Description Paragraph:
[0038] The **isolated** nucleic acids of this invention can be used to generate modified polypeptides, each having at least one characteristic of the native polypeptide. These include subfragments, deletion mutants, processing mutants, or substitution mutants, polypeptides having the same secondary structure as the binding region of the native polypeptide, and combinations thereof. Such modified polypeptides may carry the functionality of the "wild type" peptide, or may have a modified or externally regulatable functionality. Such modified polypeptides may have considerable utility in the present invention, as would be appreciated by those skilled in the art.

Detail Description Paragraph:
[0039] "Wild type", mutant and analogous polypeptides and compositions thereof may be used for making antibodies, which may find use in analyzing results of the assays described as part of this invention. The antibodies may be prepared in conventional ways either by using the subject polypeptide as an immunogen and injecting the polypeptide into a mammalian host, e.g., **mouse**, cow, goat, sheep, rabbit, etc., particularly with an adjuvant, e.g. complete Freund's adjuvant, aluminum hydroxide gel, or the like. The host may then be bled and the blood employed for **isolation** of polyclonal antibodies, or the peripheral blood lymphocytes (B-cells) may be fused with an appropriate myeloma cell to produce an immortalized cell line that secretes monoclonal antibodies specific for the subject compounds.

Detail Description Paragraph:
[0043] Chemical modifications of the phosphate backbone may be performed that reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology which

shows that this is a feasible approach. In the body, maintenance of an external concentration will typically be necessary to drive the diffusion of the modified nucleic acid sequence encoding the subject vectors into the cells of the **tissue**. Intravenous administration with a drug carrier designed to increase the circulation half-life of the subject vectors can also be used. In addition to controlling the rate of uptake, the carrier can protect the subject vectors from degradative processes.

Detail Description Paragraph:
[0045] In a preferred embodiment, the subject vectors are provided on a patch that can be adhered to the skin of the patient. This novel approach allows for an easy, noninvasive method of delivering the subject vectors to target cells and **tissues**. This patch delivery method uses the innate properties of the skin to provide prophylactic and therapeutic access to the skin's immune system. Naturally, this patch method of delivery will have certain appeal to recipients suffering from a skin disorder such as acne vulgaris. Preferably, the area intended to receive the patch can be pretreated to increase and enhance the permeability of the skin. Examples of materials that can be used to pretreat the skin include water, alcohol, hydrogels and other known permeation enchancers. Additionally, or alternatively, the patch is separately but concurrently administered with, a permeation enhancer. The patch can be adhered to the skin with known adhesives commonly used in the art.

Detail Description Paragraph:
[0047] Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to **tissues**. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Detail Description Paragraph:
[0050] The subject vectors may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, transcutaneous, intramuscular, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. A gene gun may also be utilized. Administration of DNA-coated microprojectiles by a gene gun requires instrumentation but is as simple as direct injection of DNA. A gene construct is precipitated onto the surface of microscopic metal beads. The microproj ectiles are accelerated with a shock wave or expanding helium gas, and penetrate **tissues** to a depth of several cell layers. This approach permits the delivery of foreign genes to the skin of anesthetized animals. This method of administration achieves expression of transgenes at high levels for several days and at detectable levels for several weeks. Each of these administration routes exposes the subject vectors to an accessible targeted **tissue**. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The subject vectors can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified vectors to the cell. Liposomes injected intravenously show accumulation in the **liver, lung and spleen**. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The remaining dose circulates in the blood stream for up to 24 hours.

Detail Description Paragraph:
[0055] The subject vectors may be administered utilizing an ex vivo approach whereby cells are removed from an animal, transduced with the subject vectors and reimplanted into the animal. The **liver** can be accessed by an ex vivo approach by

removing hepatocytes from an animal, transducing the hepatocytes in vitro with one or more subject vectors and reimplanting them into the animal (e.g., as described for rabbits by Chowdhury et al., Science 254:1802-1805, 1991, or in humans by Wilson, Hum. Gene Ther. 3:179-222, 1992) incorporated herein by reference.

Detail Description Paragraph:
[0058] Adenoviruses. Those skilled in the art will appreciate that for viral DNA replication and packaging of viral DNA into virion particles, only three regions of the viral DNA are known to be required in cis. These are the left inverted terminal repeat, or ITR, (bp 1 to approximately 103) the packaging signals (approximately 194 to 358 bp) (Hearing and Shenk, 1983, Cell 33: 695-703; Grable and Hearing 1992, J. Virol. 64: 2047-2056) and the right ITR. Among the regions of the viral genome that encode proteins that function in trans, two have been most important in the design and development of adenovirus vectors. These are early region 3 (E3) located between approximately 76 and 86 mu (mu=% distance from the left end of the conventionally oriented genome) and early region 1 (E1) located between approximately 1 and 11 mu. E3 sequences have long been known to be nonessential for virus replication in cultured cells and many viral vectors have deletions of E3 sequences so that the capacity of the resulting vector backbone for insertion of foreign DNA is thereby increased significantly over that allowable by the wild-type virus (Bett, A. J., Prevec, L., and Graham, F. L. Packaging capacity and **stability** of human adenovirus type 5 vectors. J. Virol. 67: 5911-5921, 1993.). E1 encodes essential functions. However, E1 can also be deleted, providing that the resulting virus is propagated in host cells, such as the 293 cell line, PER-C6 cells, 911 cells, and the like, which contain and express E1 genes and can complement the deficiency of E1(=) viruses.

Detail Description Paragraph:
[0059] Viruses with foreign DNA inserted in place of E1 sequences, and optionally also carrying deletions of E3 sequences are conventionally known as "first generation" adenovirus vectors. First generation vectors are of proven utility for many applications. They can be used as research tools for high-efficiency transfer and expression of foreign genes in mammalian cells derived from many **tissues** and from many species. First generation vectors can be used in development of recombinant viral vaccines when the vectors contain and express antigens derived from pathogenic organisms. The vectors can be used for gene therapy, because of their ability to efficiently transfer and express foreign genes in vivo, and due to their ability to transduce both replicating and nonreplicating cells in many different **tissues**. Adenovirus vectors are widely used in these applications.

Detail Description Paragraph:
[0061] It has been shown that use of hdAds can lead to prolonged transgene expression and reduced immune and inflammatory responses compared to first generation Ad vectors. HdAds retain the other beneficial properties of Ad vectors, mainly virion **stability** during vector propagation and purification, and high transduction efficiency of replicating and quiescent cells, while eliminating some of the obstacles and concerns that have been raised with respect to first-and second-generation Ads. Should transgene expression levels decrease over time, the use of hdAds of alternative serotypes may permit readministration of a vector with the identical genotype. Since vector persistence (and hence transgene expression) is influenced by immune responses to both vector and transgene, the effectiveness of vector readministration using hdAd's may ultimately depend primarily on the immunogenicity of the therapeutic gene. Accordingly, in the absence of transgene effects, the sequential use of hdAd of alternative serotype can be an effective strategy for vector readministration. Accordingly, therapeutic genes encoding products of low immunogenicity may be repeatedly administered according to the instant disclosure. In addition, in vaccine applications, in which repeat administration of a gene encoding a particular gene product against which an immune response is desired, or when administration of a second, third, fourth etc. gene is desired, ability to overcome unwanted immune responses induced by a previous

exposure to a vector is highly desirable.

Detail Description Paragraph:
[0062] Other viral vectors. Other various viral vectors can be utilized to practice the subject invention, including, but not limited to, adeno-associated virus, herpes virus, vaccinia, or an RNA virus, such as an alpha virus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Preferably, the alphavirus vector is derived from Sindbis or Semliki Forest Virus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to, Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary **tumor** virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. An alphavirus vector for use in the method of this invention comprises a recombinant alphavirus vector system which expresses the lac Z gene. Construction of this vector is described in P. Liljestrom, Current Opin. Biotechnol5(5):495-500, 1994; and P. Liljestrom et al., Biotechnology (NY) 9(12):1356-61, 1991.

Detail Description Paragraph:
[0064] A schematic diagram for the construction of the Ad5E1PBAL vector is shown in FIG. 1. To rescue the Propionibacterium acnes lipase sequences into a translatable minigene cassette, an oligonucleotide was designed containing 5' flanking restriction enzyme sites for Bam HI and Hind III, followed subsequently by a sequence coding for the consensus optimal ribosomal translation initiation site, and bases incorporating the first 30 nucleotides of the coding sequence for P. acnes lipase gene. The following is the sequence of the 5' oligonucleotide: GCGGATTCCAAGCTTGCCGCCG-CCATGAAGATCAACGCACGATTCGCCGTC. An additional oligonucleotide containing bases complementary to the 3' end of the P. acnes lipase gene flanked by residues containing stop codons to provide a translational termination signal and a restriction site Xho I was created. The sequence of the 3' oligonucleotide is: CGCCCGCTCGAGCTA-TCATGCAGCATCCGTGGTGGATACGGGCAG. Additional nucleotides were incorporated in the design of the 5' and 3' oligonucleotides to accommodate for restriction enzyme cleavage activity at blunt ends of DNA. PCR reactions were carried out using the 5' and 3' designed oligonucleotides with genomic DNA **isolated** from P. acnes bacteria.

Detail Description Paragraph:
[0065] An approximate 1 kb PCR fragment was **isolated** and subcloned by blunt end ligation into pCR-Blunt. This fragment was flanked by Bam HI and Hind III at the 5' end with an optimal consensus Kozak with 3' stop codon sequences flanked by an Xho I restriction site. The Bam HI, Hind III and Xho I restriction sites were included so that the P. acnes lipase gene could be fragmented to generate variant determinant targets since the Bam HI and Hind III sites are present within the gene. This will allow the rescue of mini-genes encoding portions of the lipase gene to be cloned into the polylinker site of pDK6. The lipase sequence was rescued from the blunt vector by Kpn I and Xho I digest and cloned into these sites in the pDK6 vector. This construction places the transgene under the control of the murine cytomegalovirus (mCMV) promoter and provides polyadneylation signals from the simian virus 40 (SV40). To obtain the resultant adenovirus vector expressing the P. acnes lipase gene, pDK6PBAL DNA was cotransfected with pBHG10 into 293 cells using standard adenovirus rescue protocols. One viral plaque was identified by restriction enzyme digest, Southern blot and by sequence to contain the P. acnes lipase gene sequence and was designated as Ad5E1PBAL vector. This recombinant vector was propagated in 293 cells and purified by cesium chloride gradient centrifugation and dialysis before use in the animal studies. All oligonucleotides used in this study were obtained from Integrated DNA Technologies, Inc., Coralville, IA.

Detail Description Paragraph:

[0066] Balb/c **mice** were purchased from the Taconic labs and bred under specific pathogen-free conditions in the McMaster University animal facility. Female **mice** were used at 8-14 wk of age. **Mice** were immunized intramuscularly with 2.times.10.sup.9 pfu in 50.mu.l saline of AdE1 Lipase (Ad5E1PBAL) or control (empty) vector (DL70-3) I.M on left hind leg. 7 days later disease was induced by injection of 100.mu.l of 1.times.10.sup.9 cfu/ml of P. acnes intramuscularly in PBS on left rear flank. All recombinant viruses were propogated and purified as described for the Ad5E1PBAL vector. Control vector DL70-3 is an Ad5 variant deleted in the E1 region. All reactions were measured by caliper sizing. FIG. 2 demonstrates that pre-immunization with lipase of P. acnes provided protections from P. acnes challenge. All work was performed in accordance with McMaster University guidelines for animal use and care. The foregoing examples are for illustration purposes only and should not be construed as limiting the scope of the subject invention.

CLAIMS:

2. The vaccine of claim 1, wherein said pathogen is P. acnes, L. **monocytogenes**, S. typhimurium, N. gonorrhoea, M. avium, M. tuberculosis, M. leprae, B. abortus, C. albicans; L. major, or combinations thereof.

15. The method of claim 1 1, wherein said pathogen is P. acnes, L. **monocytogenes**, S. typhimurium, N. gonorrhoea, M. avium, M. tuberculosis, M. leprae, B. abortus, C. albicans; L. major, or combinations thereof.

9. Document ID: US 20020022718 A1

L29: Entry 9 of 16 File: PGPB Feb 21, 2002

DOCUMENT-IDENTIFIER: US 20020022718 A1
TITLE: Genes identified as required for proliferation of E. coli

Abstract Paragraph:
The sequences of nucleic acids encoding proteins required for E. coli proliferation are disclosed. The nucleic acids can also be used to screen for homologous genes that are required for proliferation in microorganisms other than E. coli. The nucleic acids can also be used to design expression vectors and secretion vectors. The nucleic acids can be used to express proteins or portions thereof, to obtain antibodies capable of specifically binding to the expressed proteins, and to use those expressed proteins as a screen to **isolate** candidate molecules for rational drug discovery programs. The nucleic acids of the present invention can also be used in various assay systems to screen for antimicrobial agents.

Summary of Invention Paragraph:
[0011] One embodiment of the present invention is a purified or **isolated** nucleic acid sequence consisting essentially of one of the sequence of nucleotides of SEQ ID NOs: 1-93, wherein expression of said nucleic acid in a microorganism is capable of inhibiting the proliferation of a microorganism. The nucleic acid sequence may have as sequence of nucleotides complementary to at least a portion of the nucleotide sequence of the coding strand of a gene whose expression is required for proliferation of a microorganism. The nucleic acid may have a nucleotide sequence

complementary to at least a portion of the nucleotide sequence of an RNA required for proliferation of a microorganism. The nucleotide sequence of the RNA may encode more than one gene product.

Summary of Invention Paragraph:
[0012] Another embodiment of the present invention is a purified or **isolated** nucleic acid comprising a fragment of one of the nucleotide sequences of SEQ ID NOs.: 1-93, said fragment selected from the group consisting of fragments comprising at least 10, at least 20, at least 25, at least 30, at least 50 and more than 50 consecutive nucleotides of the nucleotide sequence of one of SEQ ID NOs: 1-93.

Summary of Invention Paragraph:
[0015] Another embodiment of the present invention is a purified or **isolated** nucleic acid consisting essentially of the coding sequence of one of SEQ ID NOs: 106-112, 119-122, 134-160, 164-171, 179-265, 271-273, 275, and 279-286.

Summary of Invention Paragraph:
[0018] Another embodiment of the present invention is a purified or **isolated** antisense nucleic acid comprising a nucleic acid sequence complementary to at least a portion of an intragenic sequence, intergenic sequence, sequences spanning at least a portion of two or more genes, 5' noncoding region, or 3' noncoding region within an operon comprising a proliferation-required gene whose activity or expression is inhibited by an antisense nucleic acid comprising one of SEQ ID NOs.: 1-93.

Summary of Invention Paragraph:
[0019] Another embodiment of the present invention is a purified or **isolated** nucleic acid comprising a nucleic acid having at least 70% identity to a sequence selected from the group consisting of SEQ ID NOs.: 1-93, fragments comprising at least 25 consecutive nucleotides of SEQ ID NOs.: 1-93, the sequences complementary to SEQ ID NOs.: 1-93 and the sequences complementary to fragments comprising at least 25 consecutive nucleotides of SEQ ID NOs.: 1-93 as determined using BLASTN version 2.0 with the default parameters. The nucleic acid may be from an organism selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, Listeria monocytogenes, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

Summary of Invention Paragraph:
[0022] Another embodiment of the present invention is a purified or **isolated** polypeptide comprising a polypeptide whose expression is inhibited by an antisense nucleic acid comprising one of SEQ ID NOs.: 1-93, or a fragment selected from the group consisting of fragments comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of one of the said polypeptides. The polypeptide may comprise a polypeptide comprising one of SEQ ID NOs.: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479 or a fragment comprising at least 5, at least 10, at least 20,

at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of a polypeptide comprising a sequence selected from the group consisting of SEQ ID NOs.: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479.

Summary of Invention Paragraph:
[0023] Another embodiment of the present invention is a purified or **isolated** polypeptide comprising a polypeptide having at least 25% identity to a polypeptide whose expression is inhibited by a sequence selected from the group consisting of SEQ ID NOs.: 1-93, or at least 25% identity to a fragment comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of a polypeptide whose expression is inhibited by a nucleic acid selected from the group consisting of SEQ ID NOs.: 1-93 as determined using FASTA version 3.0t78 with the default parameters. The polypeptide may have at least 25% identity to a polypeptide comprising one of SEQ ID NOs: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479 or at least 25% identity to a fragment comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of a polypeptide comprising one of SEQ ID NOs.: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479 as determined using FASTA version 3.0t78 with the default parameters.

Summary of Invention Paragraph:
[0025] Another embodiment of the present invention is a method of producing a polypeptide, comprising introducing a vector comprising a promoter operably linked to a nucleic acid encoding a polypeptide whose expression is inhibited by an antisense nucleic acid comprising one of SEQ ID NOs.: 1-93 into a cell and expressing said polypeptide. The method may further comprise the step of **isolating** said polypeptide. The polypeptide may comprise a sequence selected from the group consisting of SEQ ID NOs.: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479.

Summary of Invention Paragraph:
[0086] Another embodiment of the present invention is a purified or **isolated** nucleic acid comprising a sequence selected from the group consisting of SEQ ID NOs.: 1-93.

Detail Description Paragraph:
[0096] By "inhibit activity of a gene or gene product" is meant having the ability to interfere with the function of a gene or gene product in such a way as to decrease expression of the gene or to reduce the level or activity of a product of the gene. Agents which inhibit the activity of a gene include agents that inhibit transcription of the gene, agents that inhibit processing of the transcript of the gene, agents that reduce the **stability** of the transcript of the gene, and agents that inhibit translation of the mRNA transcribed from the gene. In microorganisms, agents which inhibit the activity of a gene can act to decrease expression of the operon in which the gene resides or alter the folding or processing of operon RNA so as to reduce the level or activity of the gene product. The gene product can be a non-translated RNA such as ribosomal RNA, a translated RNA (MRNA) or the protein product resulting from translation of the gene mRNA. Of particular utility to the present invention are antisense RNAs that have activities against the operons or genes to which they specifically hybridze.

Detail Description Paragraph:
[0111] Expression of the exogenous nucleic acid fragments in the test population of E. coli containing the expression vector library is then activated. Activation of the expression vectors consists of subjecting the cells containing the vectors to conditions that result in the expression of the exogenous nucleic acid sequences carried by the expression vector library. The test population of E. coli cells is then assayed to determine the effect of expressing the exogenous nucleic acid

fragments on the test population of cells. Those expression vectors that, upon activation and expression, negatively impact the growth of the E. coli screen population are identified, **isolated**, and purified for further study.

Detail Description Paragraph:
[0125] Recombinant DNA techniques can be used to express the entire coding sequences of the gene identified as required for proliferation, or portions thereof. The over-expressed proteins can be used as reagents for further study. The identified exogenous sequences are **isolated**, purified, and cloned into a suitable expression vector using methods well known in the art. If desired, the nucleic acids can contain the sequences encoding a signal peptide to facilitate secretion of the expressed protein.

Detail Description Paragraph:
[0135] For example, the preceding methods may be used to **isolate** nucleic acids having a sequence with at least 97%, at least 95%, at least 90%, at least 85%, at least 80%, or at least 70% identity to a nucleic acid sequence selected from the group consisting of one of the sequences of SEQ ID NOS. 1-93, 106-112,119-122, 134-160, 164-171, 179-265, 271-273, 275, and 279-286, fragments comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive nucleotides thereof, and the sequences complementary thereto. Identity may be measured using BLASTN version 2.0 with the default parameters. (Altschul, S.F. et al. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs, Nucleic Acid Res. 25: 3389-3402 (1997), the disclosure of which is incorporated herein by reference in its entirety). For example, the homologous polynucleotides may have a coding sequence which is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variants may have a substitution, deletion or addition of one or more nucleotides when compared to the nucleic acids of SEQ ID NOs: 1-93, 106-112, 119-122, 134-160, 164-171, 179-265, 271-273, 275, and 279-286 or the sequences complementary thereto.

Detail Description Paragraph:
[0136] Additionally, the above procedures may be used to **isolate** nucleic acids which encode polypeptides having at least 99%, 95%, at least 90%, at least 85%, at least 80%, at least 70%, at least 60%, at least 50%, or at least 40% identity or similarity to a polypeptide having the sequence of one of SEQ ID NOs: 299-305, 312-315, 327-353, 357-364, 372-458, 464-466, 468 and 472-479 or to a polypeptide whose expression is inhibited by a nucleic acid of one of SEQ ID NOs.: 1-93, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of the preceding polypeptides as determined using the FASTA version 3.0t78 algorithm with the default parameters. Alternatively, protein identity or similarity may be identified using BLASTP with the default parameters, BLASTX with the default parameters, or TBLASTN with the default parameters. (Alschul, S. F. et al. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs, Nucleic Acid Res. 25: 3389-3402 (1997), the disclosure of which is incorporated herein by reference in its entirety).

Detail Description Paragraph:
[0140] Hybridization of cDNA made from a sample of total cell MRNA to such an array followed by detection of binding by one or more of various techniques known to those in the art results in a signal at each location on the array to which cDNA hybridized. The intensity of the hybridization signal obtained at each location in the array thus reflects the amount of MRNA for that specific gene that was present in the sample. Comparing the results obtained for MRNA **isolated** from cells grown under different conditions thus allows for a comparison of the relative amount of expression of each individual gene during growth under the different conditions.

Detail Description Paragraph:
[0143] The present invention also contemplates additional methods for screening other microorganisms for proliferation-required genes. In this embodiment, the

conserved portions of sequences identified as proliferation-required can be used to generate degenerate primers for use in the polyrnerase chain reaction (PCR). The PCR technique is well known in the art. The successful production of a PCR product using degenerate probes generated from the sequences identified herein would indicate the presence of a homologous gene sequence in the species being screened. This homologous gene is then **isolated**, expressed, and used as a target for candidate antibiotic compounds. In another aspect of this embodiment, the homologous gene is expressed in an autologous organism or in a heterologous organism in such a way as to alter the level or activity of a homologous gene required for proliferation in the autologous or heterologus organism. In still another aspect of this embodiment, the homologous gene or portion is expressed in an antisense orientation in such a way as to alter the level or activity of a nucleic acid required for proliferation of an autologous or heterologous organism.

Detail Description Paragraph:
[0145] In another embodiment of the present invention, E. coli sequences identified as required for proliferation are transferred to expression vectors capable of function within non-E coli species. As would be appreciated by one of ordinary skill in the art, expression vectors must contain certain elements that are species specific. These elements can include promoter sequences, operator sequences, repressor genes, origins of replication,selectable marker genes, ribosomal binding sequences, termination sequences, and others. To use the identified exogenous sequences of the present invention, one of ordinary skill in the art would know to use standard molecular biology techniques to **isolate** vectors containing the sequences of interest from cultured bacterial cells, **isolate** and purify those sequences, and subclone those sequences into an expression vector adapted for use in the species of bacteria to be screened.

Detail Description Paragraph:
[0147] Following the subcloning of the identified nucleic acid sequences into an expression vector functional in the microorganism of interest, the identified nucleic acid sequences are conditionally transcribed to assay for bacterial growth inhibition. Those expression vectors found to contain sequences that, when transcribed, inhibit bacterial growth are compared to the known genomic sequence of the pathogenic microorganism being screened or, if the homologous sequence from the organism being screened is not known, it may be identified and **isolated** by hybridization to the proliferation-required E. coli sequence interest or by amplification using primers based on the proliferation-required E. coli sequence of interest as described above.

Detail Description Paragraph:
[0162] Characterization of **Isolated** Clones Negatively Affecting E. coli Proliferation

Detail Description Paragraph:
[0163] Following the identification of those inserts that, upon expression, negatively impacted E. coli growth or proliferation, the inserts were **isolated** and subjected to nucleic acid sequence determination.

Detail Description Paragraph:
[0164] The nucleotide sequences for the exogenous identified sequences were determined using plasmid DNA **isolated** using QIAPREP (Qiagen, Valencia, Calif.) and methods supplied by the manufacturer. The primers used for sequencing the inserts were 5'-TGTTTATCAGACCGCTT -3' (SEQ ID NO: 1) and 5'-ACAATTTCACACAGCCTC -3' (SEQ ID NO: 2). These sequences flank the polylinker in pLEX5BA. Sequence identification numbers (SEQ ID NOs) for the identified inserts are listed in Table I and discussed below.

Detail Description Paragraph:
Comparison Of **Isolated** Sequences to Known Sequences

Detail Description Paragraph:
[0179] The mutant allele obtained from PCR amplification is cloned into the multiple cloning site of pKO3. Directional cloning of the b2885 null allele is not necessary. The pKO3 vector has a temperature-sensitive origin of replication derived from pSC101. Therefore, clones are propagated at the permissive temperature of 30.degree. C. The vector also contains two selectable marker genes: one that confers resistance to chloramphenicol and another, the Bacillus subtilis sacB gene, that allows for counter-selection on sucrose containing growth medium. Clones that contain vector DNA with the null allele inserted are confirmed by restriction endonuclease analysis and DNA sequence analysis of **isolated** plasmid DNA. The plasmid containing the b2885 null allele insert is known as a knockout plasmid.

Detail Description Paragraph:
[0184] The above experiment is generally adequate for determining the essentiality of a gene such as b2885. However, it may be necessary or desirable to more directly confirm the essentiality of the gene. There are several methods by which this can be accomplished. In general, these involve three steps: 1) construction of an episome containing a wild type allele, 2) **isolation** of clones containing a single chromosomal copy of the mutant null allele as described above but in the presence of the episomal wild type allele, and then 3) determining if the cells survive when the expression of the episomal allele is shut off. In this case, the trans copy of wild type b2885 is made by PCR cloning of the entire coding region of b2885 and inserting it in the sense orientation downstream of an **inducible promoter** such as the E. coli lac promoter. Transcription of this allele of b2885 will be induced in the presence of IPTG which inactivates the lac repressor. Under IPTG induction b2885 protein will be expressed as long as the recombinant gene also possesses a ribosomal binding site, also known as a "Shine-Dalgarno Sequence". The trans copy of b2885 is cloned on a plasmid that is compatible with pSC101. Compatible vectors include p15A, pBR322, and the pUC plasmids, among others. Replication of the compatible plasmid will not be **temperature**-sensitive. The entire process of integrating the null allele of b2885 and subsequent plasmid excision is carried out in the presence of IPTG to ensure the expression of functional b2885 protein is maintained throughout. After the null b2885 allele is confirmed as integrated on the chromosome in place of the wild type b2885 allele, then IPTG is withdrawn and expression of functional b2885 protein shut off. If the b2885 gene is essential, cells will cease to proliferate under these conditions. However, if the b2885 gene is not essential, cells will continue to proliferate under these conditions. In this experiment, essentiality is determined by conditional expression of a wild type copy of the gene rather than inability to obtain the intended chromosomal disruption.

Detail Description Paragraph:
[0190] Antibodies capable of specifically recognizing the protein of interest can be generated using synthetic peptides using methods well known in the art. See, Antibodies: A Laboratory Manual, (Harlow and Lane, Eds.) Cold Spring Harbor Laboratory (1988). For example, 15-mer peptides having a sequence encoded by the appropriate identified gene sequence of interest or portion thereof can be chemically synthesized. The synthetic peptides are injected into **mice** to generate antibodies to the polypeptide encoded by the identified nucleic acid sequence of interest or portion thereof. Alternatively, samples of the protein expressed from the expression vectors discussed above can be purified and subjected to amino acid sequencing analysis to confirm the identity of the recombinantly expressed protein and subsequently used to raise antibodies. An Example describing in detail the generation of monoclonal and polyclonal antibodies appears in Example 7.

Detail Description Paragraph:
Production of an Antibody to an **isolated** E. coli Protein

Detail Description Paragraph:

[0194] Substantially pure protein or polypeptide is **isolated** from the transformed cells as described in Example 6. The concentration of protein in the final preparation is adjusted, for example, by concentration on a 10,000 molecular weight cut off AMICON filter device (Millipore, Bedford, Mass.), to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the protein can then be prepared as follows:

Detail Description Paragraph:
[0196] Monoclonal antibody to epitopes of any of the peptides identified and **isolated** as described can be prepared from murine hybridomas according to the classical method of Kohler, G. and Milstein, C., Nature 256:495 (1975) or any of the well-known derivative methods thereof. Briefly, a **mouse** is repetitively inoculated with a few micrograms of the selected protein or peptides derived therefrom over a period of a few weeks. The **mouse** is then sacrificed, and the antibody producing cells of the **spleen isolated**. The **spleen** cells are fused by means of polyethylene glycol with **mouse** myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as described by Engvall, E., "Enzyme immunoassay ELISA and EMIT," Meth. Enzymol. 70:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. et al. Basic Methods in Molecular Biology Elsevier, New York. Section 21-2.

Detail Description Paragraph:
[0202] Having **isolated** and expressed bacterial proteins shown to be required for bacterial proliferation, the present invention further contemplates the use of these expressed proteins in assays to screen libraries of compounds for potential drug candidates. The generation of chemical libraries is well known in the art. For example combinatorial chemistry can be used to generate a library of compounds to be screened in the assays described herein. A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining amino acids in every possible combination to yield peptides of a given length. Millions of chemical compounds theoretically can be synthesized through such combinatorial use of chemical building blocks. For example, one commentator observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds. (Gallop et al., "Applications of Combinatorial Technologies to Drug Discovery, Background and Peptide Combinatorial Libraries," Journal of Medicinal Chemistry, Vol. 37, No. 9, 1233-1250 (1994). Other chemical libraries known to those in the art may also be used, including natural product libraries.

Detail Description Paragraph:
[0206] To illustrate the screening process, the combined target and chemical compounds of the library are exposed to and permitted to interact with the purified enzyme. A labeled substrate is added to the incubation. The label on the substrate is such that a detectable signal is emitted from metabolized substrate molecules. The emission of this signal permits one to measure the effect of the combinatorial library compounds on the enzymatic activity of target enzymes. The characteristics of each library compound is encoded so that compounds demonstrating activity against the enzyme can be analyzed and features common to the various compounds identified can be **isolated** and combined into future iterations of libraries.

Detail Description Paragraph:

[0240] To provide a consistent source of cells to screen, frozen stocks of host bacteria containing the desired antisense construct are prepared using standard microbiological techniques. For example, a single clone of the organism can be **isolated** by streaking out a sample of the original stock onto an agar plate containing nutrients for cell growth and an antibiotic for which the antisense construct contains a gene which confers resistance. After overnight growth an **isolated** colony is picked from the plate with a sterile needle and transferred to an appropriate liquid growth media containing the antibiotic required for maintenance of the plasmid. The cells are incubated at 30.degree. C. to 37.degree. C. with vigorous shaking for 4 to 6 hours to yield a culture in exponential growth. Sterile glycerol is added to 15% (volume to volume) and 100 .mu.L to 500 .mu.L aliquots are distributed into sterile cryotubes, snap frozen in liquid nitrogen, and stored at -80.degree. C. for future assays.

Detail Description Paragraph:
[0242] A day prior to an assay, a stock vial is removed from the freezer, rapidly thawed (37.degree. C. water bath) and a loop of culture is streaked out on an agar plate containing nutrients for cell growth and an antibiotic to which the antisense construct confers resistance. After overnight growth at 37.degree. C., ten randomly chosen, **isolated** colonies are transferred from the plate (sterile inoculum loop) to a sterile tube containing 5 mL of LB medium containing the antibiotic to which the antisense vector confers resistance. After vigorous mixing to form a homogeneous cell suspension, the optical density of the suspension is measured at 600 nm (OD.sub.600) and if necessary an aliquot of the suspension is diluted into a second tube of 5 mL, sterile, LB medium plus antibiotic to achieve an OD.sub.600.ltoreq.0.02 absorbance units. The culture is then incubated at 37.degree. C. for 1-2 hrs with shaking until the OD.sub.600 reaches OD 0.2-0.3. At this point the cells are ready to be used in the assay.

Detail Description Paragraph:
[0278] Probes derived from the identified nucleic acid sequences of interest or portions thereof can be labeled with detectable labels familiar to those skilled in the art, including radioisotopes and non-radioactive labels, to provide a detectable probe. The detectable probe can be single stranded or double stranded and can be made using techniques known in the art, including in vitro transcription, nick translation, or kinase reactions. A nucleic acid sample containing a sequence capable of hybridizing to the labeled probe is contacted with the labeled probe. If the nucleic acid in the sample is double stranded, it can be denatured prior to contacting the probe. In some applications, the nucleic acid sample can be immobilized on a surface such as a nitrocellulose or nylon membrane. The nucleic acid sample can comprise nucleic acids obtained from a variety of sources, including genomic DNA, cDNA libraries, RNA, or **tissue** samples.

Detail Description Paragraph:
[0279] Procedures used to detect the presence of nucleic acids capable of hybridizing to the detectable probe include well known techniques such as Southern blotting, Northern blotting, dot blotting, colony hybridization, and plaque hybridization. In some applications, the nucleic acid capable of hybridizing to the labeled probe can be cloned into vectors such as expression vectors, sequencing vectors, or in vitro transcription vectors to facilitate the characterization and expression of the hybridizing nucleic acids in the sample. For example, such techniques can be used to **isolate**, purify and clone sequences from a genomic library, made from a variety of bacterial species, which are capable of hybridizing to probes made from the sequences identified in Examples 5 and 6.

Detail Description Paragraph:
[0280] The identified E. coli genes corresponding directly to or located within the operon of nucleic acid sequences required for proliferation or portions thereof can be used to prepare PCR primers for a variety of applications, including the identification or **isolation** of homologous sequences from other species, for example

S. typhimurium, E. cloacae, E. faecalis, S. pneumoniae, and K pneumoniae, which contain part or all of the homologous genes. Because homologous genes are related but not identical in sequence, those skilled in the art will often employ degenerate sequence PCR primers. Such degenerate sequence primers are designed based on conserved sequence regions, either known or suspected, such as conserved coding regions. The successful production of a PCR product using degenerate probes generated from the sequences identified herein would indicate the presence of a homologous gene sequence in the species being screened. The PCR primers are at least 10 nucleotides, and preferably at least 20 nucleotides in length. More preferably, the PCR primers are at least 20-30 nucleotides in length. In some embodiments, the PCR primers can be more than 30 nucleotides in length. It is preferred that the primer pairs have approximately the same G/C ratio, so that melting temperatures are approximately the same. A variety of PCR techniques are familiar to those skilled in the art. For a review of PCR technology, see Molecular Cloning to Genetic Engineering White, B. A. Ed. in Methods in Molecular Biology 67: Humana Press, Totowa 1997. When the entire coding sequence of the target gene is known, the 5' and 3' regions of the target gene can be used as the sequence source for PCR probe generation. In each of these PCR procedures, PCR primers on either side of the nucleic acid sequences to be amplified are added to a suitably prepared nucleic acid sample along with dNTPs and a thermostable polymerase such as Taq polymerase, Pfu polymerase, or Vent polymerase. The nucleic acid in the sample is denatured and the PCR primers are specifically hybridized to complementary nucleic acid sequences in the sample. The hybridized primers are extended. Thereafter, another cycle of denaturation, hybridization, and extension is initiated. The cycles are repeated multiple times to produce an amplified fragment containing the nucleic acid sequence between the primer sites.

Detail Description Paragraph:
[0310] Use of **Isolated** Exogenous Nucleic Acid Fragments as Antisense Antibiotics

Detail Description Paragraph:
Identification of Bacterial Strains from **Isolated** Specimens by PCR

Detail Description Paragraph:
[0340] Classical bacteriological methods for the detection of various bacterial species are time consuming and costly. These methods include growing the bacteria **isolated** from a subject in specialized media, cultivation on selective agar media, followed by a set of confirmation assays that can take from 8 to 10 days or longer to complete. Use of the identified sequences of the present invention provides a method to dramatically reduce the time necessary to detect and identify specific bacterial species present in a sample.

Detail Description Paragraph:
[0341] In one exemplary method, bacteria are grown in enriched media and DNA samples are **isolated** from specimens of, for example, blood, urine, stool, saliva or central nervous system fluid by conventional methods. A panel of PCR primers based on identified sequences unique to various species of microorganisms are then utilized in accordance with Example 12 to amplify DNA of approximately 100-200 nucleotides in length from the specimen. A separate PCR reaction is set up for each pair of PCR primers and after the PCR reaction is complete, the reaction mixtures are assayed for the presence of PCR product. The presence or absence of bacteria from the species to which the PCR primer pairs belong is determined by the presence or absence of a PCR product in the various test PCR reaction tubes.

Detail Description Paragraph:
[0342] Although the PCR reaction is used to assay the **isolated** sample for the presence of various bacterial species, other assays such as the Southern blot hybridization are also contemplated.

CLAIMS:

1. A purified or **isolated** nucleic acid sequence consisting essentially of one the sequence of nucleotides of SEQ ID NOs: 1-93, wherein expression of said nucleic acid in a microorganism is capable of inhibiting proliferation of a microorganism.

5. A purified or **isolated** nucleic acid comprising a fragment of one of the nucleotide seugence of SEQ ID NOs.: 1-93, said fragment selected from the group consisting of fragments comprising at least 10, at least 20, at least 25, at least 30, at least 50 and more than 50 consecutive nucleotides of one of the nucleotide sequences of SEQ ID NOs: 1-93.

7. The vector of claim 6, wherein said promoter is active in a microorganism selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

9. A purified or **isolated** nucleic acid consisting essentially of the coding sequence of one of SEQ ID NOs: 106-112, 119-122, 134-160, 164-171, 179-265, 271-273, 275, and 279-286.

12. A purified or **isolated** antisense nucleic acid comprising a nucleic acid sequence complementary to at least a portion of an intragenic sequence, intergenic sequence, sequences spanning at least a portion of two or more genes, 5' noncoding region, or 3' noncoding region within an operon comprising a proliferation-required gene whose activity or expression is inhibited by an antisense nucleic acid comprising one of SEQ ID NOs.: 1-93.

13. A purified or **isolated** nucleic acid comprising a nucleic acid having at least 70% identity to a sequence selected from the group consisting of SEQ ID NOs.: 1-93, fragments comprising at least 25 consecutive nucleotides of SEQ ID NOs.: 1-93, the sequences complementary to SEQ ID NOs.: 1-93 and the sequences complementary to fragments comprising at least 25 consecutive nucleotides of SEQ ID NOs.: 1-93 as determined using BLASTN version 2.0 with the default parameters.

14. The nucleic acid of claim 13, wherein said nucleic acid is from an organism selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella

boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

18. A purified or **isolated** polypeptide comprising a polypeptide whose expression is inhibited by an antisense nucleic acid comprising one of SEQ ID NOs.: 1-93, or a fragment selected from the group consisting of fragments comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of one of the said polypeptides.

20. A purified or **isolated** polypeptide comprising a polypeptide having at least 25% identity to a polypeptide whose expression is inhibited by a sequence selected from the group consisting of SEQ ID NOs.: 1-93, or at least 25% identity to a fragment comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60 or more than 60 consecutive amino acids of a polypeptide whose expression is inhibited by a nucleic acid selected from the group consisting of SEQ ID NOs.: 1-93 as determined using FASTA version 3.0t78 with the default parameters.

24. The method of claim 23, further comprising the step of **isolating** said polypeptide.

50. The method of claim 45, wherein said cell is from an organism selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

64. The method of claim 58, wherein said population is a population selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae,Listeria **monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

82. The method of claim 80 wherein said microorganism is selected from the group

consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

104. The method of claim 99, wherein said cell is selected from the group consisting of Aspergillus fumigatus, Bacillus anthracis, Burkholderia cepacia, Campylobacter jejuni, Candida albicans, Candida glabrata (also called Torulopsis glabrata), Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida krusei, Candida kefyr (also called Candida pseudotropicalis), Candida dubliniensis, Chlamydia pneumoniae, Chlamydia trachomatus, Clostridium botulinum, Clostridium difficile, Cryptococcus neoformans, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, **Listeria monocytogenes**, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella cholerasuis, Salmonella enterica, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae, **Listeria monocytogenes**, Moxarella catarrhalis, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnet, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus pneumoniae, Treponema pallidum, Yersinia pestis and any species falling within the genera of any of the above species.

124. A purified or **isolated** nucleic acid comprising a sequence selected from the group consisting of SEQ ID NOs.: 1-93.

10. Document ID: US 6890538 B1

L29: Entry 10 of 16 File: USPT May 10, 2005

DOCUMENT-IDENTIFIER: US 6890538 B1
TITLE: Immunization against herpes simplex virus

Brief Summary Text (15):
DNA vaccines (vi) have also had only limited success. There are some reports indicating that intramuscular immunisation with a plasmid carrying a gene encoding HSV antigen (glycoprotein D or B) under the control of a eukaryotic promoter is effective to induce protection in **mice** and guinea pigs against an intravaginal challenge with the virus. However, these studies also make it clear that these vaccines are ineffective in actually preventing infection. Attempts to induce protective mucosal immunity by administering DNA intranasally have similarly shown

poor results in preventing infection despite eliciting production of high titers of specific IgA antibodies.

Brief Summary Text (22):
To demonstrate rigorously that the totality of the protein produced was derived from a eukaryotic nuclear process we introduced an intron in the GFP. We found similar levels of expression of the GFP in peritoneal macrophages after the intraperitoneal inoculation of salmonellae harboring the GFP plasmid with or without the intron. Furthermore, after the oral administration we found transfected macrophages in the Peyer patches, lamina propria of the small intestine and in the **spleen**. This is the first time that expression has been conclusively demonstrated to occur only in the cells of the eukaryotic host.

Brief Summary Text (27):
In previous studies it was shown that high levels of specific secretory IgA were not enough to protect **mice** against an intravaginal infection with high or even low doses of HSV (Kuklin et al, 1997). The conclusion that antibodies at the site of mucosal infection was usually inadequate to prevent invasion came from experiments in which **mice** immunised intranasally with recombinant vaccinia expressing HSV glycoproteins were challenged vaginally with HSV. Despite high titers of both IgA and IgG vaginal antibodies against the immunising glycoprotein, following viral challenge infection occurred and virus was recovered from vaginal washes. Furthermore, the infection was confirmed because Ab response against other glycoproteins were induced, and in addition challenged animals developed secondary Ab responses to the immunising glycoprotein.

Brief Summary Text (29):
Herein, we present data that strongly support the idea that cellular immunity is responsible for the clearance of the virus. Our results show that, after intravaginal challenge, **mice** immunised with salmonellae carrying a plasmid comprising the glycoprotein D gene (pCIgD) did not develop a secondary antibody response against the immunising glycoprotein. Furthermore, in contrast to what we observed after intra-muscular immunisation with naked plasmid-DNA, immunisation with salmonellae harboring the pCIgD vector results in the absence of virus in the vaginal tract after the challenge, i.e. complete viral clearance. Cellular immunity can additionally be stimulated using cytokines such as Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interleukin-12 or other molecules that enhance the cellular immune response. According to the invention, therefore, coadministration of such molecules may assist in securing viral clearance.

Drawing Description Text (2):
FIG. 1: A) Subclass distribution of specific antibody response in serum. **Mice** were immunised with salmonella harboring pCIgD or pCI plasmid. Fifteen days after the last dose the levels of antibodies against epitopes 8-23 was determined by ELISA at a dilution 1/100. B) Development of DTH reation in SL7207pCIgD or SL7207pCI immunised **mice**. Each group of six **mice** was immunised as was described in methods. For the DTH assay, each **mouse** was injected with either 10.sup.8 UV inactivated HSV (titrated before inactivation) in the right ear pinna or BHK extract in the left ear pinna. *p<0.01. C) Expression of IL-2R by CD4+ **spleen** cells after in vitro stimulation with UV inactivated HSV (left panel) or with mock antigen (right panel). A gate on CD4+ T cell was performed and the histograms for IL-2R expression were displayed. Values represent the mean.+-.SD from five animals per group. For the statistical analysis the Mann Whitney U test was used.

Drawing Description Text (3):
FIG. 2: **Mice** were immunised with salmonella. Fifteen days after the last immunisation **spleen**, Peyer patches (PP) and ileal lymph node (ILN) cell suspensions were restimulated in vitro with UV inactivated HSV or mock antigen over a period of 24 hs. Frequencies of cytokine-producing cells in **spleen** (A), PP (B) and ILN were measured by ELISPOT assay. Pooled data from three experiments (three **mice** per group

in each experiment) are presented. Bars represent the mean.+-.SE. *p<0.001, **p<0.05.

Drawing Description Text (4):
FIG. 3: Profile of cytokines released by HSV, mock or peptide 291-306 stimulated cells from **spleen**. 10.sup.7 cells/ml were cultured per quadruplicate in 24 well plates and after 24 and 48 hs of culture, supernatantes were harvested and the level of cytokines was determined by sandwich ELISA. Bars represent the mean.+-.SE. *p<0.001.

Drawing Description Text (5):
FIG. 4: Protective immune response after oral genetic immunisation. Groups of five **mice** were immunised as was described in methods with salmonella harboring pCIgD or pCI plasmid. Fifteen days after the last immunisation **mice** were injected with 3 mg of DP. Five days following the administration of DP **mice** were challenged intravaginally with 5.times.10.sup.6 PFU of HSV2 MS strain. Numerical scores were assigned to specific disease signs using the following scale: 0, no symptoms; 1, mild inflammation; 2, moderate swelling; 3, severe inflamation; 4, paralysis and 5, death. Daily mean lesion score was calculated by dividing the sum of a group's lesion scores by the number of observations. Graphics represent pooled data from three independent experiments with five **mice** per group in each experiment.

Drawing Description Text (6):
FIG. 5: Expression of GFP in peritoneal macrophages. Three **mice** per group were inoculated intraperitoneally with 5.times.10.sup.6 salmonellae harboring one of the following plasmids: pCIgD, pCIGFP or pCIGFPint. Two days after the inoculation cells were collected from the peritoneal cavity and the expression of GFP was determined by flow cytometric analysis. Characterisation of the cell subset expressing GFP was done by two colour fluorometric analysis after staining with PE-MAC3 or PE-CD19. No expression of GFP was observed in the CD19+ B cells. The values are the mean.+-.SD from four **mice** per group.

Drawing Description Text (7):
FIG. 6: Characterisation of in vivo S. typhimurium-mediated DNA gene transfer. **Mice** were fed with salmonella harboring one of the following plasmids: pCI, pCIGFP or pCIGFPint. Five days after the last dose the expression of GFP was determined by flow cytometry in Peyer patches (A,B,C), lamina propria of the small intestine (D,E,F) and in the **spleen** (G,K,I). Cell subsets expressing GFP were identified by two-colour fluorocytometric analysis staining with PE-anti CD19 (B cells). PE-anti CD3 (T cells) or PE-MAC3 (macrophages). Only the macrophage subset expressed GFP. No expression of GFP was found in B or T cells. The values showed represent the mean.+-.SD from four **mice**).

Detailed Description Text (16):
The attenuating mutations can be either constitutively expressed or under the control of **inducible promoters, such as the temperature** sensitive heat shock family of promoters (Neidhardt et al, supra), or the anaerobically **induced nirB promoter** (Harborne et al, Mol. Micro., 6:2805-2813 (1992)), or repressible promoters, such as uapA (Gorfinkiel et al, J. Biol. Chem., 268:23376-23381 (1993)) or gev (Stauffer et al, J. Bact., 176:6159-6164 (1994)).

Detailed Description Text (100):
Mice

Detailed Description Text (101):
Female BALB/c **mice**, 6 to 8 weeks of age, were purchased from Harlan (Italy) and maintained at the International Centre of Genetic Engineering and Biotechnology under standard conditions according to Institutional Guidelines.

Detailed Description Text (111):

HSV-2 MS strain (ATCC No. VR-540) DNA used as template for polymerase chain reactions (PCR), was prepared from nucleocapsids **isolated** from BHK cells. For the construction of the eukaryotic expression vector pCIgD a 1.2 kb fragment encoding the gD precursor gene was amplified by PCR using the following primers: forward, GTTCGGTCATAAACTGCATTGCGAACCACTAGTCG (SEQ ID No. 1); reverse, CCTAGTTTCCCTCCTTCTAGACTCCCTTTATGCGG (SEQ ID No. 2). The PCR product was cloned in the EcoRI-XCba site of the eukaryotic expression vector pCI and completely sequenced. The GFP was amplified by PCR using as template the CMV2 tracer plasmid (Invitrogen) and then subcloned in the EcoRI-Xba site of the expression vector pCI (pCIGFP). Intron 1 of the human alpha globin gene (117 bp) was introduced into the GFP by PCR-directed mutagenesis at the position 283. The PCR product was cloned as was described above for the GFP (pCIGFPint).

Detailed Description Text (115):
Bacteria were grown overnight until they reached stationary phase. They were harvested by centrifugation and resuspended in PBS with 5% sodium bicarbonate. **Mice** received three groups of doses at 15 days intervals by feeding them with the bacterial suspension using a flexible cannula. Each group of doses consisted in three doses at 2-days intervals of 5-10.times.10.sup.7 recombinant S. typhimurium AroA strain harboring one of the plasmids described. As control was used the bacterial transformed with the vector pCI alone. For protection studies, 15 days after the last dose of salmonellae, immunised **mice** were challenged by intravaginal instillation of HSV-2 MS strain. In order to synchronise the estrus cycle, the immunised **mice** were injected subcutaneously with Depo-Provera (DP) (Upjohn Co., Kalamazoo, Mich.) at a concentration of 3 mg per **mouse** in 100 .mu.l of distilled water (Parr et al, 1994). Five days after the administration of DP, the animals were anesthesiated with Avertin and the vaginal cavity was washed with PBS previously of the instillation of 5.times.10.sup.6 PFU of HSV in 20 .mu.l. The **mice** were examined daily for vaginal inflammation, neurologic illness and death. The severity of disease was scored 1 to 5 (0, no symptoms; 1, mild inflammation; 2, moderate swelling; 3, severe inflammation; 4, paralysis and 5, death) (Overall et al, 1975). Vaginal washes were collected at different time points after intravaginal challenge by pipetting 200 .mu.l of PBS in the vaginal cavity. The samples were filtered by 0.45 .mu.m filters and stored at -80.degree. C. until titered.

Detailed Description Text (117):
To evaluate the levels of specific total IgG or IgG subclasses in serum and in vaginal washes, standard indirect ELISA was employed. Levels of total IgG, IgG1 and IgG2a were determined using affinity-purified rabbit anti-**mouse** specific for .gamma., .gamma.1 or .gamma.2a respectively and as second antibody an affinity purified goat anti rabbit IgG HRPO conjugate (Zymed Lab. Inc. San Francisco, Calif.) was used. As antigen for attachment to the plates the whole gD and two synthetic peptides (aa 8-23, and aa 222-252) corresponding to the main neutralising in vitro epitopes (Cohen et al, 1984; Nicola et al, 1998) were used.

Detailed Description Text (121):
Fifteen days after the last dose of salmonellae, **mice** were sacrificed and **spleen**, Peyer patches, mesenteric lymph node and ileal lymph node were removed and cultured for 24 hr in presence of inactivated HSV or mock antigen. After this, cells were added to 96 well nitrocellulose bottom plates (Millipore, HA) precoated with anti cytokine antibody. Cells were cultured for 24 hr and removed. After the addition of the second anti-cytokine monoclonal antibody conjugated with biotin, the HRPO conjugated streptavidin was dispensed. The spots were developed with the substrate AEC (Fujihashi et al, 1993).

Detailed Description Text (123):
Expression of IL-2R responding to Ag stimulation in vitro was determined by FACS analysis. **Spleen** cells (1.times.10.sup.7 /ml) cultured for 96 hs in the presence of 1/20 dilution of UV inactivated HSV or mock antigen, were collected and doubly

stained with PE-labelled anti-**mouse** CD4 and with FITC-labelled monoclonal antibodies: anti-**mouse** IL-2R (Pharmingen) for 30 min at 4.degree. C. Cells were washed twice with medium and a two-colour immunofluorescence analysis using a FACScalibur (Becton Dickinson) was performed, with gates set by forward angle light and side scatter. Gates were adjusted to include the discrete mononuclear population and exclude dead cells and debris, and 20.times.10.sup.3 cells were analysed per sample.

Detailed Description Text (127):
Mice were fed with SL7207 harboring the either pCIGFP, pCIGFPint or pCIgD vectors, following the schedule of administration described above Lamina propria and Peyer patches cell suspensions were prepared as was previously described by Franco et al (1998). GFP-expressing cells in PP, MLN and LP were detected by flow cytometry 5 days after the last dose. The phenotype of the GFP+ cells was determined by double-fluorescence analysis after staining with anti CD3-PE, anti CD19-PE, anti MAC3-PE or anti CD11c-PE in the presence of Fc blocking reagent (Pharmingen). In other experiments one dose 4.times.10.sup.6 SL7207 harboring the plasmids mentioned above were administered intraperitoneally to 3 groups of **mice**. Two days later peritoneal cells were harvested and GFP+ cells were detected by double fluorescence analysis after staining with MAC3-PE or CD19-PE.

Detailed Description Text (130):
Mice orally transgen immunised using attenuated salmonella (strain SL7207) harboring the pCIgD plasmid, elicited a weak serum antibody immune response. The reactivity of the antibodies was mainly against the epitope 8-23 whereas very low levels against the epitope 222-252 were detected. Analysis of the IgG subclass distribution in the immune sera indicated IgG2a as the principal isotype, but also IgG1 was evident (FIG. 1A). A very weak serum IgA antibody response was also observed with a pattern of reactivity similar to that described for IgG. Furthermore, no antibody reactivity was observed in the vaginal washes when whole gD or peptides were attached to the plates.

Detailed Description Text (132):
When **spleen** cells from **mice** immunised with SL7207 harboring the pCIgD or the pCI plasmid were cultured in the presence of inactivated HSV or mock antigen, an increase in the expression of IL-2R was observed in the CD4 subset (FIG. 1C). This was taken as an indicator that the transgene immunisation using salmonella results in a specific CD4 activation. To determine whether antigen-specific cell-mediated responses could be detected in vivo, delayed type hypersensitivity (DTH) reaction was measured 15 days after the last dose of salmonella. Significant DTH response was induced in the immunised **mice** indicating that a Th1 immune response was present (FIG. 1B). The pattern of cell-mediated immune response was directly analysed by enumerating the number of cells in Peyer patches, ileal lymph nodes and **spleen**, which produce Th1- or Th2-type cytokines upon secondary stimulation in vitro with inactivated HSV or with synthetic peptide (aa 291-306). **Mice** immunised with SL7207 harboring the pCIgD vector showed a dramatical increase in the IFN-.gamma. secreting cells in **spleen**, ileal lymph node (ILN) and Peyer patches (PP) (FIG. 2). However, a weak increment in the number of specific IL-2 secreting cells was observed. Surprisingly, we observed an increase of the IL-4 producing cells in the **spleen** of immunised **mice**, which could be responsible of the IgG1 immune response observed in serum. When the pattern of cytokines was analysed in the supernatant of cultured lymphocytes after in vitro stimulation, the results were in line with those described above (FIG. 3). However, we did not find significant differences in the levels of IL-4 among the different groups of **mice**. This may indicate that the analysis at single cell level could be more sensitive than the measure of cytokines in the supernatants.

Detailed Description Text (134):
Fifteen days after the last dose **mice** were challenged intravaginally with 5.times.10.sup.6 PFU. Animals were followed for signs of disease, and vaginal

washes were collected for virus titration. All immunised animals were protected and survived challenge, whereas within 13 days post challenge all the controls died (FIG. 4). During the observation period, non-immunised animals showed herpetic lesions. On the other hand, controls showed inflammation, swelling and paralysis prior to death (FIG. 4). One point of the fundamental importance was to determine whether the immune response developed in the immunised **mice** was enough to prevent viral invasion. If the immunity mounted in immunised animals resulted in total exclusion of the virus, then the virus should be rapidly removed from vaginal **tissues** and the animals should show no evidence for a systemic secondary immune response to HSV. Indeed no virus could be recovered from vaginal washing of immunised **mice** (Table 1). Furthermore, when the level of serum antibodies against gD or HSV was determined no secondary immune response was found.

Detailed Description Text (136):
One point to address was to determine whether the immune response observed was against a protein synthesised in vivo by the eukaryotic cells or otherwise it resulted from the expression of the antigen in the bacterial carrier. To assess this, the intron 1 of the human alpha globin gene was introduced in the GFP so that, to obtain a fluorescent protein it needs a splicing process that only can occur in the nucleus. The flow cytometry analysis of the peritoneal macrophages obtained from **mice** inoculated with one dose of salmonellae harboring the pCIGFP or the pCIGFPint showed similar expression of the GFP (FIG. 5). This result unanswerably confirms that a real gene transfer from bacteria with a de novo synthesis by the host cell have occurred.

Detailed Description Text (138):
To determine what kind of cells express the transgene delivered by the orally administered salmonellae carrier and in which organs these cells can be found, **mice** were fed with the SL7207 strain harboring the pCIGFP, the pCIGFPint or the pCI vectors. The flow cytometric analysis showed the expression of the GFP in cells from PP, **spleen** and LP of the small intestine. The phenotype of cells expressing GFP was characterised by double fluorescence analysis. Only macrophages and dendritic cells expressed GFP (FIG. 6). No CD19+ or CD3+ lymphocytes were positive for GFP expression.

Detailed Description Paragraph Table (1):
TABLE 1 Resistance to HSV challenge in SL7207 pCIgD immunised **mice** and controls No. of **mice** Log10 of viral titer Serum IgG anti gD titers survived/no. of at day post challenge (aa 8-23) Immunisation **mice** challenged 1 3 5 prechallenge postchallenge SL7207pCI 0/15 0.9 .+-. 0.2 4.5 .+-. 1.2 4.8 .+-. 0.8 ND ND (7/8) (8/8) (8/8) SL7207pCIgD 15/15 0 0 0 150 .+-. 80 180 .+-. 40 (0/7) (0/7) (0/7)

Other Reference Publication (11):
Bourne et al, "DNA immunization confers protective immunity on **mice** challenged intravaginally with herpes simplex virus type 2", Vaccine 14(13):1230-1234 (1996).

Other Reference Publication (14):
Chabalgoity et al, "A Salmonella typhimurium htrA live vaccine expressing multiple copies of a peptide comprising amino acids 8-23 of herpes simplex virus glycoprotein D as a genetic fusion to tetanus toxin fragment C protects **mice** from herpes simplex virus infection", Molecular Microbiology 19(4):791-801 (1996).

Other Reference Publication (17):
McDermott et al, "T Lymphocytes in Genital Lymph Nodes Protect **Mice** from Intravaginal Infection with Herpes Simplex Virus Type 2", The Journal of Infectious Diseases 159(3):460-466 (1989).

CLAIMS:

1. A method of generating an immune response to a herpes simplex virus (HSV) in a

human or animal host, said method comprising: (a) providing an immunogenic compositions; wherein said composition is comprised of an invasive but attenuated or non-pathogenic bacterium selected from the group consisting of Salmonella, Shigella, **Listeria** and E. coli bacteria; said bacterium is comprised of a coding sequence encoding a herpes simplex virus (HSV) antigen selected from the group consisting of glycoprotein D, glycoprotein H, glycoprotein B and ICP27; and said coding sequence is comprised within an expression construct and operably linked to one or more regulatory sequences and (b) administering said composition to said host such that said bacterium invades a host cell of said host selected from the group consisting of macrophages and dendritic cells and said expression construct is transferred to said host cell where said regulatory sequences direct expression of said coding sequence, and said HSV antigen is transcribed and translated in said host cell without introduction of an antimicrobial agent to lyse the bacterium to generate said immune response against HSV.

7. A method according to claim 1, wherein said bacterium is a Salmonella, Shigella or **Listeria** bacterium.

18. A method of generating a protective immune response to a herpes simplex virus (HSV) in a human or animal host, said method comprising: (a) providing an immunogenic composition; wherein said composition is comprised of an invasive but attenuated or non-pathogenic bacterium selected from the group consisting of Salmonella, Shigella, **Listeria** and E. coli bacteria; said bacterium is comprised of a coding sequence encoding a herpes simplex virus (HSV) antigen selected from the group consisting of glycoprotein D, glycoprotein H, glycoprotein B and ICP27; and said coding sequence is comprised within an expression construct and operably linked to one or more regulatory sequences and (b) administering said composition to said host such that said bacterium invades a host cell of said host selected from the group consisting of macrophages and dendritic cells and said expression construct is transferred into said host cell where said regulatory sequences direct expression of said coding sequence, and said HSV antigen is transcribed and translated in said host cell without introduction of an antimicrobial agent to lyse the bacterium to generate said prophylactic immune response against HSV.

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L29: Entry 11 of 16 File: USPT Jul 6, 2004

DOCUMENT-IDENTIFIER: US 6759241 B1
**** See image for** Certificate of Correction **
TITLE: Adjuvant comprising a lipopolysaccharide antagonist

Abstract Text (1):
LPS preparations, **isolated** from gram negative bacterial strains that contain at least one mutation in at least one of the htrB and msbB genes, and methods and therapeutics related thereto. The LPS preparations display both LPS antagonist and adjuvant activities.

Brief Summary Text (17):
There is now evidence that mutations in htrB and msb may influence the biosynthesis of lipid A (Karow, et al., 1991, J. Bacteriol., 173:741; Karow and Georgopoulos,

1992, J. Bacteriol., 174:702). These mutants are temperature sensitive and LPS **isolated** from these mutants stains less intensely on silver-stain gels (Karow and Georgopoulos, 1992, supra). The basis for the temperature-sensitive growth phenotype of the htrB and msb mutants has remained cryptic (Karow and Georgopoulos, 1992, supra). There was speculation that these mutants produce defective lipid A precursors (Karow and Georgopoulos, 1992, supra). This was based on the observation that ammonium cationic compounds enabled these mutants to grow in non permissive temperatures (Karow and Georgopoulos, 1992, supra). These investigators proposed that the ammonium cationic compounds influenced the intermolecular interaction between LPS molecules in the outer membrane. This observation is supported by a report showing that an htrB mutant of Haemophilus influenzae produces modified LOS structures (Lee, et al., 1995, Infect. Immun., 63:818; Lee, et al., 1995, In: Abstracts of the American Society for Microbiology, ASM Washington D.C., p.206 (B-234)). Later studies showed direct evidence that htrB and msb mutants could produce substantially pure non-pyrogenic LPS (see PCT International Publication Nos. WO 97/18837 and WO 99/15162, the teachings of which are incorporated herein by reference).

Brief Summary Text (18):
Other lipid A precursor structures **isolated** from E. coli mutants that are defective in the biosynthesis of lipid A are described in Raetz, et al., 1985, supra; Kovach, et al., 1990, J. Exp. Med., 172:77; Golenbock, et al., 1991, J. Biol. Chem., 266:19490; Golenbock, et al., 1988, Antimicrob. Agents Chemother., 32:37; Clementz, et al., 1996, J. Biol. Chem., 271:12095; Clementz, et al., 1997, J. Biol. Chem., 272:10353; Garrett, et al., 1998, J. Biol. Chem., 273:12457; Kitchens, et al., 1992, J. Exp. Med., 176:485; Kitchens, and Munford, 1995, J. Biol. Chem., 270:9904; Munford and Hunter, 1992, J. Biol. Chem., 267:10116; Rietschel, et al., 1994, supra; Ulmer, et al., 1992, Infect. Immun., 60:5145; Ulmer, et al., 1992, Infect. Immun., 60:3309; Wang, et al., 1990, FEMS Micro. Immunol., 2:179; Wang, et al., 1991, Infect. Immun., 59:4655 64.

Brief Summary Text (21):
More recently, E. coli msbB mutants have been shown to produce LAS and LPS preparations **isolated** from E. coli msbB mutants were shown to possess LPS antagonist activity (Clementz, et al., 1997, supra; Somerville, et al., 1996, J. Clin. Invest., 97:359). Similarly, other investigators have shown that E. coli hirB and msbB mutants (Clementz, et al., 1996, supra; Clementz, et al., 1997, supra; Hone, et al., 1998, J. Human Virol., 1:251), Salmonella htrB mutants (Sunshine, et al., 1997, J. Bacteriol., 179:5521; Jones, et al., 1997, Infect. Immun., 65:4778), and msbB mutants (Low, et al., 1999, Nature Biotech., 17:37; Khan, et al., 1998, Mol. Microbiol., 29:571), and Haemophilus htrB mutants (Lee, et al., 1995, J. Biol. Chem., 270:27151) produce defective lipid A structures, predominated by LA4 and LA5, that are non pyrogenic and display LPS antagonist activity.

Brief Summary Text (27):
The invention relates to methods and compositions comprising an adjuvant which is both an LPS antagonist and is non-pyrogenic. In a preferred embodiment, the non-pyrogenic LPS antagonist adjuvant is **isolated** from a gram negative bacterial strain that contains at least one mutation in at least one of the htrB and msbB genes.

Brief Summary Text (28):
In one aspect, the invention features an adjuvant comprising an LPS antagonist, wherein said LPS antagonist is **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes. In one embodiment, the LPS antagonist has reduced pyrogenicity. In a preferred embodiment, the LPS antagonist has substantially reduced pyrogenicity. In a more preferred embodiment, the LPS antagonist in non-pyrogenic. In a preferred embodiment, pyrogenicity is determined by measuring the levels of indicators of pyrogenicity or inflammation, such as, for example, IL-1.beta., IL-6 or TNF.alpha., in a cell, extracellular medium, or a subject. In a most preferred embodiment, the LPS antagonist elicits no detectable

TNF.alpha. activity when contacted with a cell or administered to a subject.

Brief Summary Text (31):
In another embodiment, the invention provides a pharmaceutical preparation comprising a vaccine antigen, a pharmaceutically effective amount of an LPS antagonist, **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, and a pharmaceutically acceptable carrier. The vaccine antigen may be any vaccine antigen, such as e.g., a polysaccharide, a protein or a nucleic acid. In an embodiment of the invention, the vaccine antigen is derived from a viral pathogen selected from the group consisting of orthomyxoviruses, retroviruses, herpesviruses, lentiviruses, rhabdoviruses, picornoviruses, poxviruses, rotavirus and parvoviruses. Exemplary antigens are influenza virus, RSV, EBV, CMV, herpes simplex virus, human immunodeficiency virus, rabies, poliovirus and vaccinia, human immunodeficiency virus antigens Nef, p24, gp120, gp41, Tat, Rev, and Pol; T cell and B cell epitopes of gp120; the hepatitis B surface antigen; rotavirus antigens, such as VP4 and VP7; influenza virus antigens such as hemagglutinin or nucleoprotein; and herpes simplex virus thymidine kinase.

Brief Summary Text (34):
In additional embodiments of the invention the vaccine antigen is derived from a **tumor** antigen selected from the group consisting of prostate specific antigen, TAG-72, carcinoembrionic antigen (CEA), MAGE-1, tyrosinase, and mutant p53 antigen; the CD3 receptor on T cells; an autoimmune antigen; or the IAS .beta. chain. Alternatively, the invention can be practiced with a vaccine antigen such as an immuno-stimulatory molecule selected from the group consisting of M-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-.gamma..

Brief Summary Text (36):
In another aspect, the invention features methods for preparing and using an adjuvant as described herein comprising an LPS antagonist, wherein said LPS antagonist is **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes.

Brief Summary Text (37):
In another aspect, the invention features methods for preparing and using a vaccine comprising a vaccine antigen and a pharmaceutically effective amount of an LPS antagonist **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, and a pharmaceutically acceptable carrier.

Detailed Description Text (3):
Prior to the present invention, LPS antagonists had proven to be poor adjuvants. An unexpected and surprising element of the present invention, therefore, is the finding that LPS preparations **isolated** from gram negative bacterial strains that contain at least one mutation in at least one of the hirB and msbB genes display both LPS antagonist and adjuvant activities. Accordingly, one embodiment of the present invention provides a novel adjuvant comprising an LPS antagonist. In particular, the present invention provides compositions comprising an LPS antagonist **isolated** from a gram negative bacterial strain that contains at least one mutation in at least one of the htrB and msbB genes. The invention also provides methods for enhancing the efficacy of a vaccine in a subject, comprising administering to the subject one or more antigens, against which an immune response is desired, with one or more LPS antagonists.

Detailed Description Text (9):
The terms "biological activity", "bioactivity", "activity" and "biological function", when referring to a LPS or lipid A preparation, and which are used interchangeably herein, includes a biological response of a cell when contacted with LPS or lipid A, e.g., an increase in the production of a cytokine. A biological activity of an LPS or lipid A preparation also includes an effect of the

LPS or lipid A on an organism, e.g, an increase in body temperature, i.e., fever; or toxicity, B-lymphocyte mitogenesis, macrophage activation, interferon production, **tumor** regression, peripheral vascular collapse ("endotoxic" shock), pulmonary hypertension, pulmonary edema, disseminated intravascular coagulopathy and pyrogenicity. An LPS bioactivity can also include the modulation of gene expression of LPS-responsive genes, e.g., IL-1, IL-6, RANTES, or TNF-.alpha. and the consequent serum levels of their gene products. Other biological activities of LPS include activation of cellular and humoral immunity, enhancement of the tumoricidal activity of macrophages and stimulation of the release of numerous inflammatory mediators such as **tumor** necrosis factor, arachidonic acid metabolites, complement components, reactive oxygen intermediates, nitric oxide, hydrolytic enzymes, thromboxane, prostacyclin, and platelet activating factor, chemoattractants, interleukin (IL)-8, leukotriene B4, and proteases. LPS bioactivity also includes phosphorylation of specific proteins including MAP kinases, e.g., MAPK1, MAPK4, and p38.

Detailed Description Text (16):
The term "**isolated**" as applied to LPS or lipid A molecules, refers LPS or lipid A which has been **isolated** from other bacterial components, in particular from bacterial glycoproteins.

Detailed Description Text (18):
The term "lipid A analog" refers to a lipid A molecule having essentially the same molecular structure as wildtype lipid A (FIG. 2) and having the same biological activity as wildtype lipid A. Lipid A analogs can be modified to be shortened or condensed, e.g., the carbon backbone may be shortened to a 5 carbon backbone. In another example, glucosamine residues are substituted with galactosamine residues. In yet another example, a synthetic analog contains a 2-deoxy-2-aminogluconate in place of the glucosamine-1-phosphate at the reducing end. In another illustrative embodiment, a synthetic analog bears a galacturonic acid moiety instead of a phosphate at position 4'. Lipid A analogs can be prepared from lipid A **isolated** from a bacterium, e.g., by first determining the structure of the **isolated** lipid A and synthesizing analogs thereof.

Detailed Description Text (19):
The term "lipid A derivative" is a lipid A analog that can be obtained from a wildtype lipid A by chemical derivation. For example, a lipid A derivative is a lipid A molecule that is obtained by deacylation of a wildtype lipid A molecule, e.g., by alkali treatment. Lipid A derivatives can be prepared from lipid A **isolated** from bacteria, as well as from synthetic lipid A molecules.

Detailed Description Text (22):
The term "LPS analog" refers to a molecule having essentially the same molecular structure as wildtype LPS (FIG. 1) and having the same biological activities as wild-type LPS. LPS analogs can be prepared from LPS **isolated** from a bacterium, e.g., by first determining the structure of the **isolated** LPS and synthesizing analogs thereof.

Detailed Description Text (23):
The term "LPS derivative" is an LPS analog that can be obtained from a wildtype LPS molecule by chemical derivation. Lipid A derivatives can be prepared from lipid A **isolated** from bacteria, as well as from synthetic lipid A molecules.

Detailed Description Text (25):
The term "mixture of LPS" or "mixture of lipid A", e.g., a heterologous mixture, refers to a composition comprising more than one LPS antagonist or derivative or analog thereof, whether synthetic or **isolated** from a microorganism. No specific amount, ratio, or number of different LPS antagonists is necessary to form a heterologous mixture. For example, such a mixture may contain two or more different types of lipid A molecules. In an illustrative embodiment, growth of an E. coli

strain that is defective in htrB or in both htrB and msbB in permissive conditions (30.degree. C.) yields a heterologous mixture of penta-acylated and some hexa-acylated forms of lipid A (Clementz et al., 1997, JBC 272:10353).

Detailed Description Text (29):
The term "non-human animals" includes any animal that can be treated or used in testing the present invention, including mammals such as non-human primates, rodents, sheep, dogs, cows, pigs, chickens, as well as amphibians, reptiles, etc. Preferred non-human animals are selected from the primate family or rodent family (e.g., rat and mouse).

Detailed Description Text (46):
The term "wildtppe lipid A" refers to lipid A having the molecular structure of a lipid A molecule **isolated** from a wildtype microorganism, i.e., a microorganism that has not been mutated in a laboratory by the hand of man. A preferred wildtype lipid A is a lipid A from an E. coli bacteria, e.g., W3110 (see Examples).

Detailed Description Text (47):
The term "wildtype LPS" refers to LPS having the molecular structure of an LPS molecule **isolated** from a wildtype microorganism, i.e., a microorganism that has not been mutated in a laboratory by the hand of man. A preferred wildtype LPS is an LPS from an E. coli bacteria, e.g., W3110, which can be purchased from Ribi ImmunoChem Research, Inc., Hamilton, Mont. (see Examples).

Detailed Description Text (55):
The mutations can be either constitutively expressed or under the control of **inducible promoters, such as the temperature** sensitive heat shock family of promoters (Neidhardt, et al., supra), or the anaerobically-**induced nirB promoter** (Harborne, et al., 1992, Mol. Micro., 6:2805) or repressible promoters, such as uapA (Gorfinkiel, et al., 1993, J. Biol. Chem., 268:23376) or gcv (Stauffer, et al., 1994, J. Bact, 176:6159). Selection of the appropriate promoter will depend on the host bacterial strain and will be obvious to those skilled in the art.

Detailed Description Text (79):
A straight forward approach to identifying the optimal temperature for the culture of a particular bacterial strain is to grow the bacteria over a range of culture temperatures, **isolate** LPS from each culture (as described herein below) and measure the LPS antagonist activity of the LPS produced (as described in Hone, et al., 1998, supra). In this manner, culture temperatures can be identified that result in the production of non-pyrogenic or substantially lowered pyrogenic LPS antagonist by the bacterial strains.

Detailed Description Text (90):
While one embodiment of the current invention provides an adjuvant comprised of LPS antagonist **isolated** from mutant gram negative bacterial strains using the above extraction procedure, in another embodiment the adjuvant can be prepared by **isolating** the lipid A fraction of the LPS preparation.

Detailed Description Text (91):
Procedures for **isolating** the lipid A fraction from LPS are well known in the art (see, e.g., Garrett, et al., 1997, supra; Garrett, et al., 1998, supra; Clementz, et al., 1996, supra; Clementz, et al., 1997, supra). For illustrative purposes, the lipid A fraction can be **isolated** from an LPS antagonist preparation by mild acid hydrolysis in 1% SDS at pH 4.5. The lipid A fraction can then be **isolated** by conventional DEAE-cellulose column chromatography techniques (Garrett, et al., 1997, supra; Garrett, et al., 1998, supra; Clementz, et al., 1996, supra; Clementz, et al., 1997, supra).

Detailed Description Text (95):
Various methods well known in the art can be used to determine the molecular

structure of an LPS or lipid A molecule **isolated** and purified from bacteria. Exemplary methods are described, e.g, in U.S. Pat. No. 5,648,343, in which the molecular structure of lipid A from Phizobium leguminosarum was determined. In particular, this patent describes methods for determining the glycosyl composition, fatty acid composition, glycosyl linkage analysis, and phosphate content of lipid A preparations. Such methods may involve NMR Spectroscopy and high resolution mass spectrometry, e.g., fast atom bombardment mass spectrometry (FAB-MS).

Detailed Description Text (103):
Glycosyltransferase enzymes for synthesizing the compositions of the invention can be obtained commercially or may be derived from biological fluids, **tissue** or cell cultures. Such biological sources include, but are not limited to, pig serum and bovine milk. Glycosyltransferases that catalyze specific glycosidic linkages may routinely be **isolated** and prepared as described in International Patent Publication No. WO 93/13198 (published Jul. 8, 1993). Alternatively, the glycosyltransferases can be produced through recombinant or synthetic techniques known in the art (For review, see Wong et al., 1994, Enzymes in Synthetic Organic Chemistry, Pergamon Press, Volume 12, pp. 275-279).

Detailed Description Text (109):
Also included within the scope of the present invention are LPS and lipid A molecules which are differentially modified during or after synthesis, or after **isolation** from bacteria, e.g., to reduce their pyrogenicity. In specific embodiments, the LPS and lipid A molecules are treated by alkaline hydrolysis or acyloxyacyl hydrolase. Any of numerous chemical modifications may be carried out by known techniques, such as acylation, deacylation, formylation, oxidation, reduction, etc.

Detailed Description Text (110):
It is also within the scope of this invention, to synthesize analogs of lipid a having one or mere acyloxyacyl groups removed. Lipid A, either chemically synthesized or **isolated** from a gram negative microorganism may be treated with acyloxyacyl hydrolase in order to achieve or enhance the non-pyrogenic properties of the preparation. Acyloxyacyl hydrolase hydrolyzes the ester bonds between non-hydroxylated fatty acids and the 3-hydroxy functions of 3-hydroxy fatty acids bound in ester or amide linkages to glucosamine disaccharide of lipid A.

Detailed Description Text (111):
It is further within the scope of this invention, to synthesize analogs of lipid A and LPS having one or more non-hydroxylated fatty acids removed. Lipid A or LPS either chemically synthesized or **isolated** from a gram negative microorganism may be deacylated in order to achieve or enhance the substantially reduced or absent pyrogenicity of the preparation.

Detailed Description Text (114):
Various tests can be used to demonstrate that a compound is an LPS antagonist (see Examples section herein). For example, various amounts of the test compound can be incubated with a cell having LPS receptors and wildtype LPS, e.g., LPS from E. coli W3110, in conditions under which, but for the presence of the test compound, the wildtype LPS binds to the LPS receptor and induces LPS biological activities. One or more biological activities of LPS are then monitored and compared to those obtained when cells and wildtype LPS are incubated in the absence of the test compound. Optionally, the test comprises an LPS binding protein. The presence of a decrease in one or more biological activities of wildtype LPS in the presence of the compound relative to the absence of the compound indicates that the test compound is an LPS antagonist. A similar test in which the effect of a test compound on at least one biological activity of LPS is determined can also be performed in vivo, e.g., in a test animal such as a **mouse**. For example, the blood level of TNF can be measured in **mice** to which wildtype LPS and one of several doses of a test compound is administered (as described, e.g., in U.S. Pat. No.

5,158,939).

Detailed Description Text (118):
In another illustrative embodiment, a peripheral blood mononuclear cell (PBMC) activation assay can be used to assess the pyrogenic and LPS antagonist activities of an LPS antagonist preparation. These methods are well documented (Theofan, et al., 1994, J. Immunol., 152:3623; Fagan, et al., 1994, J. Immunol., 153:5230; Verhasselt, et al., 1997, J. Immunol., 158:2919; Colotta, et al., 1992, J. Immunol., 148:760; Mackensen, et al., 1992, Eur. Cyto. Net., 3:571; Eggesbo, et al., 1994, Cytokine, 6:521; Hone, et al., 1998, supra). Briefly, human PBMCs are **isolated** from whole blood and suspended at a density of 5.times.10.sup.5 per ml in complete medium (CM; RPMI containing 10 .mu.g/ml of pyruvate and glutamine, 100 .mu.g/ml of penicillin and streptomycin, and 10% (v/v) endotoxin-free human AB serum (Life Technologies)). The PBMCs are then placed into 48 well flat-bottom culture plates (Costar), and stimulated with an LPS antagonist preparation (to measure the pyrogenic activity) or mixtures of an LPS antagonist and Re LPS (Sigma) to measure LPS antagonist activity, as described (Kovach, et al., 1990, supra; Golenbock, et al., 1991, supra; Golenbock, et al., 1988, supra; Kitchens, et al., 1992, supra; Kitchens and Munford, 1995, supra; Munford and Hunter, 1992, supra; Rietschel, et al., 1994, supra; Ulmer, et al., 1992, supra; Ulmer, et al., 1992, supra; Wang, et al., 1990, supra; Wang, et al., 1991, supra; Qureshi, et al., 1991, supra; Qureshi, et al., 1991, supra; Zuckerman and Qureshi, 1992; Hone, et al., 1998, supra). In positive control wells the PBMCs are stimulated with comparable doses of E. coli Re LPS (Sigma) in place of the LPS antagonist and in negative wells the PBMCs remain unstimulated. Culture supernatants are collected 8, 24 or 48 hours after addition of the LPS and TNF-.alpha. IL-1 .beta. and/or other cytokine levels in the culture supernatants are quantitated by commercially available capture ELISAs (R & D Systems).

Detailed Description Text (119):
The efficacy of the adjuvant to enhance an immune response against an antigen can be determined, e.g., by examining the presence and/or the extent of a humoral (antibody) response and/or cell mediated immunity. Assays for measuring humoral and cell mediated responses are known in the art and are also described in Example 3 and, e.g., in PCT/US98/26291 (WO 99/29728). For example, a composition of the present invention containing an LPS antagonist may be tested in **mice** for the ability to enhance an antibody response to an antigen and the delayed-type hypersensitivity (DTH) response, measured by an increase in footpad swelling after inoculation in the footpad of the test animal, as compared to the measurements in an animal to which the same composition without the LPS antagonist was administered. Each animal in the test group may receive the amount of antigen combined with different amounts of the LPS antagonist. Serum samples are then obtained from each animal after the final inoculation, and the serum is analyzed for the presence of antibodies against the antigen using methods known in the art, e.g., ELISA. DTH responses to the antigen can be measured after the final inoculation (e.g., within 1-7 days). An increase in serum antibodies against the antigen and/or an increase in footpad swelling in the animals having received the antigen together with an LPS antagonist relative to the responses in animals having received the antigen alone indicates that the LPS antagonist is an adjuvant.

Detailed Description Text (131):
Alternatively, the vaccine antigen may be a **tumor**-, transplantation-, or autoimmune specific antigen. These latter vaccine antigens may be given alone or in combination with one or more **tumor**-, transplantation-, or autoimmune-specific antigens.

Detailed Description Text (138):
Examples of **tumor** specific antigens include but are not restricted to prostate specific antigen (Gattuso, et al., 1995, Human Pathol., 26:123), TAG-72 and carcinoembrionic antigen (CEA) (Kris, et al., 1999, Cancer Res., 59:676; Guadagni,

et al., 1994, Int. J. Biol. Markers, 9:53), MAGE-1 and tyrosinase (Coulie, et al., 1993, J. Immunother., 14:104); mutant p53 antigen (Mayordomo, et al., 1996, J. Exp. Med., 183:1357). Recently it has been shown in **mice** that immunization with non-malignant cells expressing a **tumor** antigen provides a vaccine effect by inducing an immune response that clears malignant **tumor** cells displaying the same antigen (Koeppen, et al., 1993, Anal. N.Y. Acad. Sci., 690:244).

Detailed Description Text (140):
Examples of autoimmune antigens include IAS .beta. chain (Topham, et al., 1994, Proc. Natl. Acad. Sci., USA, 91:8005-8009). Vaccination of **mice** with an 18 amino acid peptide from IAS .beta. chain has been demonstrated to provide protection and treatment to **mice** with experimental autoimmune encephalomyelitis (Topham, et al., 1994, supra).

Detailed Description Text (162):
The methods and compositions of the invention may be used as a vaccine in a subject in which immunity for the antigen(s) is desired. Such antigens can be any antigen known in the art to be useful in a vaccine formulation. The methods and compositions of the present invention can be used to enhance the efficacy of any vaccine known in the art. The vaccine of the present invention may be used to enhance an immune response to infectious agents and diseased or abnormal cells, such as, but not limited to, bacteria, parasites, fungi, viruses, **tumors**, and cancers. The compositions of the invention may be used to either treat or prevent a disease or disorder amenable to treatment or prevention by generating an immune response to the antigen provided in the composition. In one preferred embodiment, the antigen(s) are proteins, fragments or derivatives, including truncation isoforms thereof, encoded by any genes of the HIV genome including the env, gag, pol, nef, vif, rev, and tat genes. In a more preferred embodiment, the antigen is an HIV-associated gp120 protein.

Detailed Description Text (165):
The subjects to which the present invention is applicable may be any mammalian or vertebrate species, which include, but are not limited to, cows, horses, sheep, pigs, fowl (e.g., chickens), goats, cats, dogs, hamsters, **mice**, rats, monkeys, rabbits, chimpanzees, and humans. In a preferred embodiment, the subject is a human.

Detailed Description Text (167):
Toxicity and therapeutic efficacy of the LPS antagonist can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD.sub.50 /ED.sub.50. LPS antagonists which exhibit large therapeutic indices are preferred. While LPS antagonists that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected **tissue** in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

Detailed Description Text (174):
The LPS antagonist may be administered alone or in combination with other molecules known to have a beneficial effect, such as to enhance the immune response to an antigen (e.g., adjuvant activity), molecules capable of **tissue** repair and regeneration and/or inhibiting inflammation. Examples of useful cofactors include chemokines (see WO 99/29728). Other useful cofactors include symptom-alleviating cofactors, including antiseptics, antibiotics, antiviral and antifungal agents and analgesics and anesthetics. In addition, substances that enhance the **stability** and/or activity of the LPS antagonist may be co-administered, either together or sequentially, with the LPS antagonist. In yet another embodiment, the co-factor is an antagonist to any deleterious activity or side-effect of the LPS antagonist.

Detailed Description Text (175):
The LPS antagonist also may be associated with means for targeting the LPS antagonist to a desired **tissue**. For example, an antibody or other binding protein that interacts specifically with a surface molecule on the desired target **tissue** cells may be used. Such targeting molecules further may be covalently associated to the LPS antagonist, e.g., by chemical crosslinking, or by using standard genetic engineering means to create, for example, an acid labile bond such as an Asp-Pro linkage. Useful targeting molecules may be designed, for example, using the simple chain binding site technology disclosed, for example, in U.S. Pat. No. 5,091,513.

Detailed Description Text (179):
In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Other suitable delivery systems include microspheres which offer the possibility of local noninvasive delivery of drugs over an extended period of time. This technology utilizes microspheres of precapillary size which can be injected via. a coronary catheter into any selected part of the body, without causing inflammation or ischemia. The administered therapeutic is slowly released from these microspheres and taken up by surrounding **tissue** cells.

Detailed Description Text (181):
In clinical settings, a gene delivery system for the gene encoding a therapeutic or vaccine antigen can be introduced into a patient in conjunction with the LPS antagonist by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or **tissue**-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter or by stereotactic injection.

Detailed Description Text (192):
6.1.2 **Isolation** and Purification of LPS

Detailed Description Text (193):
The liquid culture was harvested by centrifugation at 7000.times.g for 10 minutes, washed once in 250 ml endotoxin-free irrigation saline (Baxter) and the weight of the bacterial pellet was determined. The pellets was then resuspended in endotoxin-free water (Baxter) at a final density of 2% w/v.+-.0.25%. Subsequently, LPS was **isolated** by two cycles of hot-water phenol extraction. In short, the bacterial suspension was heated to 70.degree. C. to which an equal volume of pre-warmed phenol was added and mixed for 15 minutes at 70.degree. C. The mixture was cooled to 25.degree. C. and then centrifuged at 18,000.times.g for 15 minutes. Following this centrifugation the aqueous phase was removed, placed into dialysis tubing (SpectraPor) and dialyzed against running distilled H.sub.2 O overnight. The retentate was then placed into fresh 50 ml polypropylene tubes and treated with RNaseA (100 mg/ml) at 37.degree. C. for 1 hour, followed by DNaseI (50 mg/ml and 5 mM MgCl.sub.2) at 37.degree. C. for 1 hour, followed by Pronase (250 mg/ml) at 37.degree. C. for 1 hour. Then EDTA was added to a final concentration of 5 mM and the hot-water phenol extraction procedure described above was repeated. Following dialysis, the retentate was centrifuged at 20,000.times.g for 15 minutes al

4.degree. C. The supernatants were transferred to fresh Beckman 50Ti tubes and the LPS was pelleted by centrifugation at 110,000.times.g for 2 hours at 4.degree. C. The supernatants were discarded and the pellets were vacuum dried. The LPS preparation was evaluated for DNA and protein contamination by SDS-PAGE and silver stain, BCA protein estimate assay and UV spectrophotometry.

Detailed Description Text (195):
To confirm that MLK986 LPS possesses LPS antagonist activity, MLK986 LPS (cultured at 37.degree. C.) at 1 .mu.g/ml was mixed with varying amounts of W3110 LPS (also obtained from bacteria grown at 37.degree. C., and **isolated** as described above for MLK986 LPS) from 1 ng/ml to 100 ng/ml and these mixtures were used to stimulate human peripheral blood mononuclear cells (PBMCs). W3110 LPS (also cultured at 37.degree. C.) from 1 ng/ml to 100 ng/ml alone and W3110 LPS (10 ng/ml) mixed with synthetic lipid IV.sub.A (1 .mu.g/ml) were used as controls. For this, PBMCs were obtained from 50 ml of whole blood and resuspended in complete medium (CM; RPMI containing pyruvate, glutamine, PenStrep, and 10% endotoxin-free human AB serum (Life Technologies) at a density of 6.times.10.sup.6 PBMCs/ml. CM containing W3110 LPS, MLK986 LPS or synthetic lipid IV.sub.A was placed into duplicate wells of a 96-well flat bottom culture plate (Costar) at double the target final concentration. An equal volume of CM containing the PBMCs then was added to these wells and the culture plates were incubated at 37.degree. C. in 5% CO.sub.2 for 8 hr. The supernatants were then removed and stored at 70.degree. C. Quantitation of TNF.alpha., IL-1.beta., and IL-6 in these culture supernatants was achieved by capture ELISA (R&D Systems).

Detailed Description Text (200):
Vaccine preparations containing a peptide antigen and an LPS antagonist were prepared in the following manner. Each vaccine preparation contained 50 mg of the vaccine peptide (herein designated "Hep-Tat") that corresponds to the heparin-binding domain of the HIV-1 regulatory protein Tat and is comprised of the following sequence (GLGIS YGRKKRRQR; SEQ ID NO: 1). The peptide antigen was prepared synthetically as a Multiple Antigen Peptide (MAPS;Genosys). To formulate the preparations comprised of Hep-Tat and LPS antagonist (purified as outlined above), 50 mg of Hep-Tat was combined with a range of LPS antagonist doses (from 1 mg to 10 mg). The LPS antagonist was **isolated** from MLK986 cultured at 37.degree. C. as described in. Example 1. The dose of peptide was chosen from preliminary experiments in which 50 mg of Hep-Tat was found to be marginally immunogenic when formulated with Alum (Pierce) as an adjuvant.

Detailed Description Text (201):
Groups of three BAL Blc **mice** were injected intraperitoneally with a single dose of a given preparation of Hep-Tat and LPS antagonist (i.e., primary vaccination). To determine the immunogenicity of the antigen in the absence of LPS antagonist, a group of BALB/c **mice** were vaccinated with 50 .mu.g Hep-Tat formulated-alone. For comparative purposes, an additional group of **mice** was vaccinated with 50 .mu.g Hep-Tat formulated with Alum, as described (Coligan, et al., 1994, supra).

Detailed Description Text (202):
To measure the immunogenicity of each formulation, venous blood was collected from the **mice** in each group before and on day 14 after primary vaccination, as described (Coligan, et al., 1994, supra). Serum was then obtained from each of these blood samples using standard techniques (Coligan, et al., 1994, supra). On day 21 after the primary vaccination, **mice** were given a booster vaccination containing the same dose of Hep-Tat and LPS antagonist or alum as was given in the primary vaccination. Seven days after the boost, venous blood was collected from the **mice** in each, and sera were obtained as outlined above. Each of the serum samples from individual groups were pooled and the level of Hep-Tat-specific IgG was ascertained by ELISA. In short, plastic 96 well ELISA plates (Immunol 2 Dynatech) were coated with Hep-Tat, by adding 50 .mu.g of Hep-Tat at 10 .mu.g/ml suspended in PBS (Phosphate Buffer Saline) pH 7.4 in each well, and incubating overnight at 4.degree. C. The

plates were subsequently washed to remove unbound peptide and then 100 .mu.l of blocking solution (comprised of 1% (w/v) Bovine Serum Albumin (BSA; Sigma) suspended in PBS) was added to each well. After incubating the plates for 1 hour at 25.degree. C., the blocking solution was removed and 3-fold serial dilutions of the sera (suspended in PBS containing 1% BSA and 0.5% Tween-20 (Sigma) from the vaccinated **mice** were added to each well. The plates were then incubated for a further 1 hour at 25.degree. C. and then washed thoroughly 4 times with PBS containing 0.5% Tween-20. Then, 100 .mu.l of goat anti-**mouse** IgG antibody conjugated to horse radish peroxidase (from Sigma and diluted 1:2000 in PBS containing 1% BSA) was added to each well. The plates were incubated for 1 hour at 25.degree. C. The plates were then, washed thoroughly a further 4 times with PBS containing 0.5% Tween-20 to remove unbound anti-**mouse** antibody and then 100 .mu.l of substrate was added to each well. The plates were then incubated for a further 30-60 minutes. Further degradation of the substrate was terminated by adding stopping solution. The result of the ELISA were obtained by reading the optical density of each well using a Molecular Dynamics ELISA plate reader. The data, shown in Table 2, are presented as dilution of the sera to achieve the 50% saturation.

Detailed Description Text (208):
Vaccine preparations containing an LPS antagonist and a protein antigen can be prepared in the following manner. Each vaccine preparation contained 5 .mu.g of fully glycosylated endotoxia-free HIV-1.sub.MN gp120 (Virostat Cat. No. 8919, Lot AM735). Preparations comprised of gp120 and LPS antagonist were formulated essentially as outlined in Example 2 above except that each dose contained 5 .mu.g of gp120 combined with a range of LPS antagonist doses (from 1 .mu.g to 10 .mu.g). The LPS antagonist was **isolated** from MLK986 cultured at 37.degree. C. as described above. The dose of gp120 was chosen from preliminary experiments that determined this dose to be marginally immunogenic when formulated with Alum as an adjuvant.

Detailed Description Text (209):
Groups of 3 BALB/c **mice** were given a single dose of a given preparation of gp120 and LPS antagonist subcutaneously (primary vaccination). To determine the immunogenicity of the antigen in the absence of LPS antagonist, a group of BALB/c **mice** were vaccinated with 5 .mu.g gp 120 formulated alone. For comparative purposes, an additional group of **mice** were vaccinated with 5 .mu.g of gp120 formulated with Alum, as described (Coligan, et al., 1994, supra).

Detailed Description Text (210):
To measure the immunogenicity of each formulation, sera were collected from the **mice** in each group before and on day 14 after primary vaccination, as described in Example 2 above. On day 21 after the primary vaccination, **mice** were given a booster vaccination containing 10 .mu.g of gp120 formulated with matched doses of LPS antagonist, as was given in the primary vaccination. Ten and 17 days after the boost, venous blood was collected from the **mice,** and sera were obtained as outlined above. The level of anti-gp120 IgG in pooled serum samples was determined by ELISA as described in Example 2 above, except that gp120 was used in place of Hep-Tat in the first coating step. The results of the ELISA were obtained by reading the optical density of each well using a Molecular Dynamics ELISA plate reader and are shown in Table 3 below. The data are presented as dilution of the sera to achieve 50% saturation.

Detailed Description Text (212):
To further measure the adjuvant activity of the LPS antagonist, the level of HIV-1 gp120-specific T cell proliferation was quantitated using a conventional .sup.3 H-thymidine-incorporation assay (Wu, et al., 1997, AIDS Res. Human Retrovirus, 13:1187). **Spleen** cells were harvested 28 days after the final vaccination and stimulated with bovine serum albumin (10 .mu.g/ml) or purified recombinant gp 120 (10 .mu.g/ml). The stimulated cells and matched unstimulated control cells were incubated for three days at 37.degree. C. in a 5% CO.sub.2 incubator. At this time, .sup.3 H-thymidine was added to each well and the cells were incubated for a

further 48 hours, as described in Wu, et al., 1997, supra).

Detailed Description Text (219):
Vaccine preparations containing a vaccine antigen and an adjuvant were prepared in the following manner. Each vaccine preparation contained 5 mg of endotoxin-free plasmid DNA, herein designated pSF128.2, which expresses HIV-1.sub.Ba-L gp140 (Reitz, et al., 1994, AIDS Res. Hum. Retroviruses, 10:621; Ivey-Hoyle, et al., 1991, Proc. Natl. Acad. Sci. USA, 88:512) under the control of the CMV immediate early promoter. HIV-1.sub.Ba-L gp140 is an important HIV-1 vaccine target. This envelope derivative is expressed in a soluble form, yet it forms an oligomeric structure that contains discontinuous epitopes that are present on the native envelope structure (Stamatos, et al., 1998, J. Virol., 72:9656). Preparations comprising pSF128.2 and LPS antagonist were formulated essentially as outlined in Example 2 above except that each dose contained 5 mg of pSF128.2 combined with a range of LPS antagonist doses (from 1 mg to 30 mg; see Table 4). The LPS antagonist was **isolated** from MLK986 cultured at 37.degree. C., as described above.

Detailed Description Text (220):
Groups of 3 BALB/c **mice** were given a single dose of a given preparation of pSF 128.2 and LPS antagonist intramuscularly (primary vaccination). Four weeks after vaccination the **mice** were boosted with 10 .mu.g of purified recombinant gp140 also formulated with LPS antagonist. The amount of LPS antagonist used in the booster vaccines was matched to the amount used in the DNA vaccine; the dose of the booster antigen used was insufficient by itself to induce a measurable immune response against gp140. Thus, this strategy provided an efficient method for measuring the priming effect of the DNA vaccine formulations. To determine the immunogenicity of the pSF128.2 in the absence of LPS antagonist, a group of 3 BALB/c **mice** was vaccinated with .mu.g pSF128.2 and boosted 4 weeks later with 10 .mu.g of gp140 formulated alone. For comparative purposes, an additional group of **mice** was vaccinated with 5 .mu.g of pSF 128.2 DNA formulated with 50 .mu.g of monophosphoryl-lipid A (MPLA; Ribi Immunochem Research), as described (Sasaki, et al., 1997, Infection and Immunity 65:3520-3528) and boosted 4 weeks later with 10 .mu.g of gp140 formulated with 50 .mu.g of MPLA.

Detailed Description Text (221):
To measure the immunogenicity of each formulation, sera were collected from the **mice** in each group before and on days 14 and 60 after primary vaccination, as described in Example 2 above. The level of anti-HIV-1 gp140 Env IgG in pooled serum samples was determined by ELISA as described in Example 2 above, except that purified HIV-1.sub.Ba-L gp140 was used in the place of Hep-Tat in the first coating step. The results of the ELISA were obtained by reading the optical density of each well using a Molecular Dynamics ELISA plate reader and are shown in Table 5.

Detailed Description Text (227):
Vaccine preparations containing an LPS antagonist and a polysaccharide were prepared in the following manner. The antigen used in this Example consists of endotoxin-free Salmonella typhi Vi polysaccharide. Antibodies directed against this polysaccharide vaccine antigen have been shown to be protective in animals and humans. Vi polysaccharide has been used in field trials to provide protection against typhoid fever (Robbins and Robbins, 1984, supra; Klugman., et al., 1996, supra). Preparations comprised of Vi and LPS antagonist were formulated essentially as outlined in Example 2 above, except that each group received the following doses: 0.001, 0.01, 0.1 or 1 mg of Vi combined with a range of LPS antagonist doses (from 1-30 .mu.g), using a conventional checkerboard approach as described above (see Table 6, groups 1.1-4.4). The LPS antagonist was **isolated** from MLK986 cultured at 37.degree. C. as described above.

Detailed Description Text (228):
Groups of 3 BALB/c **mice** were injected subcutaneously with 0.1 ml of one of the above formulations, comprised of a given dose of Vi and LPS antagonist. To

determine the immunogenicity of Vi in the absence of LPS antagonist, groups of 3 BALB/c **mice** were vaccinated with Vi formulated alone (see Table 5, groups 1.5-4.5). For comparative purposes, groups of **mice** were vaccinated with analogous doses of Vi polysaccharide formulated with 10 .mu.g of MPLA (Ribi Co.) (see Table 5, groups 1.6-4.6), as directed by the manufacturer.

Detailed Description Text (229):
To measure the immunogenicity of each formulation, sera were collected from the **mice** in each group before and on days 14 and 28 after vaccination, as described in Example 2 above. The level of anti-gp120 IgG in pooled serum samples was determined by ELISA as described in Example 2 above, except that Vi (10 .mu.g/ml) was used in place of Hep-Tat in the first coating step. The results of the ELISA were obtained by reading the optical density of each well using a Molecular Dynamics ELISA plate reader and showed that the LPS antagonist displays adjuvant properties when formulated with a Vi polysaccharide vaccine.

Detailed Description Text (234):
To assess the agonist activity of each preparation for the induction of .beta.-chemokine secretion by human PBMCs we conducted a PBMC activation assay. Briefly, human PBMCs were obtained from whole blood as described (Crowley, et al. 1996, J. Immunol, 156:2004). The PBMCs (5.times.10.sub.5) were suspended in complete medium (CM; RPMI (Lifetechnologies, Bethesda Md.) containing 10 .mu.g/ml of pyruvate and glutamine, 100 .mu.g/ml of penicillin and streptomycin, and 10% (v/v) endotoxin-free human AB serum (Life Technologies)) and placed into 48 well flat-bottom culture plates (Costar). CM containing the LPS preparations was then added to these wells at a concentration of 100 ng/ml and the PBMCs were incubated at 37.degree. C. in 5% CO.sub.2. Culture supernatants were collected 24 hr after addition of the LPS preparations and MIP-1 a, MIP-1 .beta. and RANTES levels in the culture supernatants were quantitated by capture ELISAs (R&D Systems). The results of this assay, shown in FIG. 3, demonstrate that the culture conditions substantially influence the magnitude of the agonist activity of the LPS produced by strain NILK986 for the induction of .beta.-chemokine secretion by human PBMCs. In this experiment the .beta.-chemokine-inducing agonist activity of the LPS **isolated** from MLK986 was strongest when this strain was cultured in low magnesium medium, at pH 7.4, and at 30.degree. C. and 37.degree. C. In a subsequent experiment the .beta.-chemokine-inducing agonist activity of MLK986 LPS was significantly diminished in media buffered at pH 6.0. Hence, pH is an additional culture medium parameter that is useful for the purpose of identifying the specific culture conditions that generates non-pyrogenic LPS or lipid A with strong chemokine agonist activity. In such instances, the pH of the media could be varied from 4.0 to 9.0 depending on the particular strain being assessed.

Other Reference Publication (8):
Berglund, et al., "Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in **mice.**" 1999, Vaccine, 17:497-507.

Other Reference Publication (16):
Coulie, et al., "Genes coding for **tumor** antigens recognized by human cytolytic T lymphocytes." 1993, J . Immunother., 14:104-109.

Other Reference Publication (17):
Davis, et al., "CpG DNA is a potent enhancer of specific immunity in **mice** immunized with recombinant hepatitis B surface antigen." 1998, J. Immunol., 160:870-876.

Other Reference Publication (25):
Fairweather, et al., "Oral vaccination of **mice** against tetanus by use of a live attenuated salmonella carrier." 1990, Infect. Immun., 58:1323-26.

Other Reference Publication (30):
Frenkel, et al., "The **isolation,** characterization and cloning of a globin-like,

host-protective antigen from the excretory-secretory products of Trichostrongylus colubriformis." 1992, Mol. Biochem. Parasitol., 50:27-36.

Other Reference Publication (38):
Golenbock, et al., "Elimination and **tissue** distribution of the monosaccharide lipid A precursor, lipid X, in **mice** and sheep." 1988, Antimicrob. Agents Chemother., 32:37-41.

Other Reference Publication (41):
Grant, et al., "Differential plasmid rescue from transgenic **mouse** DNA into Escherichia coli methylation-restriction mutants." 1990, Proc. Natl. Acad. Sci. USA., 87:4645-4649.

Other Reference Publication (44):
Guadagni, et al., "In vitro and in vivo regulation of human **tumor** antigen expression by human recombinant interferons: a review." 1994, Int. J. Biol. Markers, 9:53-60.

Other Reference Publication (52):
Hogan and Vogel, "Production of **tumor** necrosis factor by rIFN-y-primed C3H/HeJ (Lpsd.sup.d) macrophages requires of the presence of lipid A-associated proteins." 1988, J. Immunol., 141:4196-4202.

Other Reference Publication (58):
Ivey-Hoyle, et al., "Envelope glycoproteins from biologically diverse **isolations** of immunodeficiency viruses have widely different affinities for CD4." 1991, Proc. Natl. Acad. Sci. USA, 88:512-516.

Other Reference Publication (62):
Karow, et al., "**Isolation** and characterization of the Escherichia coli htrB gene, whose product is essential for bacterial viability above 33C in rich media." 1991, J. Bacteriol., 173:741-750.

Other Reference Publication (64):
Karow and Georgopoulos, "**Isolation** and characterization of the Escherichia coli msbB gene, a multicopy suppressor of null mutations in the high-temperature requirement gene htrB." 1992, J. Bacteriol., 174:702-710.

Other Reference Publication (65):
Kass, et al., "Induction of protective host immunity to carcinoembryonic antigen (CEA), a self-antigen in CEA transgenic **mice**, by immunizing with a recombinant vaccinia-CEA virus." Cancer Research, 59:676-683.

Other Reference Publication (68):
Kirikae, et al., "CD14 is not involved in Rhodobacter sphaeroides diphosphoryl lipid A inhibition of **tumor** necrosis factor alpha and nitric oxide induction by taxol in murine macrophages." 1995, Infect. Immun., 63:486-497.

Other Reference Publication (78):
Kovach, et al., "Lipid iV.sub.A Inhibits synthesis and release of **tumor** necrosis factor induced by Lipopolysaccharide in human whole blood ex vivo." 1990, J. Exp. Med., 172:77-84.

Other Reference Publication (79):
Kurane, et al., "Cytokines as an adjuvant to **tumor** vaccines: efficacy of local methods of delivery." 1997, Ann. Surg. Oncol., 4:579-585.

Other Reference Publication (90):
Low, et a., "Lipid A mutant salmonella with suppressed virulence TNFa induction retain **tumor**-targeting in vivo." 1999, Nature Biotech., 17:37-41.

Other Reference Publication (98):
Mayordomo, et al., "Therapy of murine **tumors** with p53 wild-type and mutant sequence peptide-based vaccines." 1996, J. Exp. Med., 183:1357-1365.

Other Reference Publication (103):
Morishita, et al., "Stable expression of human **tissue**-type plasminogen activator regulated by B-actin promoter in three human cell lines: HeLa, WI-38 VA13 and KMS-5." 1991, Biochem. Biophys. Acta., 1909:216-222.

Other Reference Publication (119):
Quershi, et al., "Diphosphoryl Lipid A obtained from the nontoxic lipopolysaccharide of Rhodopseudomonas sphaeroides is an endotoxin antagonist in **mice**." 1991, Infect. Immun., 59:441-444.

Other Reference Publication (122):
Raetz, et al., "**Isolation** and characterization of eight lipid A precursors from a 3-deoxy-D-manno-octyloxonic Acid-deficient mutant of Salmonella typhimurium." 1985, J. Bio. Chem., 260:16080-16088.

Other Reference Publication (124):
Reitz, et al., "Partial envelope sequences from some of the earliest **isolates** of HIV-1." 1994, AIDS Res. Hum. Retroviruses, 10:621-623.

Other Reference Publication (130):
Roberts, et al., "Recombinant P.69/pertactin: immunogenicity and protection of **mice** against Bordetella pertussis infection." 1992, Vaccine, 29:43-48.

Other Reference Publication (149):
Tabatabai, et al., "Monophosphoryl lipid A-induced immune enhancement of Brucella abortus salt-extractable protein and lipopolysaccharide vaccines in BALB/c **mice**." 1992, Am. J. Bet. Res., 53:1900-1908.

Other Reference Publication (159):
Ugozzoli, et al., "Intranasal immunication of **mice** with herpes simplex virus type 2 recombinant gD2: the effect of adjuvants on mucosal and serum antibody responses." 1998, Immunol., 93:563.

Other Reference Publication (172):
Wu, et al., "Induction of mucosal and systemic responses against human immunodeficiency virus type 1 glycoprotein 120 in **mice** after oral immunication with a single dose of a salmonella-HIV vector." 1997, AIDS Res. Human Retroviruses, 13:1187-1194.

Other Reference Publication (175):
Zuckerman and Qureshi, "In vivo inhibition of lipopolysaccharide-induced lethality and **tumor** necrosis factor synthesis by Rhodobacter sphaeroides diphosphoryl lipid A is dependent on corticosterone induction." 1992, Infect. Immun., 60:2581-2587.

CLAIMS:

1. A vaccine preparation comprising (i) a pharmaceutically effective amount of a substantially pure LPS antagonist **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, or an analog or derivative thereof, wherein the LPS antagonist has reduced pyrogenicity relative to an LPS antagonist **isolated** from the wildtype bacterium, (ii) a vaccine antigen, which is not **isolated** from the gram negative bacterium, and (iii) a pharmaceutically acceptable carrier.

18. The vaccine preparation of claim 17, wherein the bacterial pathogen is selected

from the group consisting of Mycobacterium spp., Helicobacter pylori, Salmonella spp., Shigella spp., E. coli, Rickettsia spp., **Listeria** spp., Legionella pneumoniae, Pseudomonas spp., Vibrio spp., and Borellia burgdorferi.

22. The vaccine preparation of claim 1, wherein the vaccine antigen elicits an immune reaction against a **tumor** antigen.

23. The vaccine preparation of claim 22, wherein the **tumor** antigen is selected from the group consisting of prostate specific antigen, TAG-72, carcinoembrionic antigen (CEA), MAGE 1, tyrosinase, and mutant p53 antigen.

28. A method for preparing a vaccine preparation of claim 1, comprising combining a substantially pure LPS antagonist **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, or an analog or derivative thereof, wherein the LPS antagonist has reduced pyrogenicity relative to an LPS antagonist **isolated** from the wildtype bacterium; a vaccine antigen that is not derived from the bacterium; and a pharmaceutically acceptable carrier.

29. A method for inducing an immune response against an antigen in a subject, comprising administering to the subject a pharmaceutically effective amount of an LPS antagonist **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, or an analog or derivative thereof, wherein the LPS antagonist has reduced pyrogenicity relative to an LPS antagonist **isolated** from the wildtype bacterium and a vaccine antigen that is not derived from the bacterium, to thereby induce an immune response against the vaccine antigen in the subject.

45. The method of claim 44, wherein the bacterial pathogen is selected from the group consisting of Mycobacterium spp., Helicobacter pylori, Salmonella spp., Shigella spp., E. coli, Rickettsia spp., **Listeria** spp., Legionella pneumoniae, Pseudomonas spp., Yibrio spp., and Borellia burgdorferi.

49. The method of claim 29, wherein the vaccine antigen elicits an immune reaction against a **tumor** antigen.

50. The method of claim 29, wherein the **tumor** antigen is selected from the group consisting of prostate specific antigen, TAG-72, carcinoembrionic antigen (CEA), MADE-1, tyrosinase, and mutant p53 antigen.

55. A kit for inducing an immune reaction against an antigen in a subject, comprising a (i) pharmaceutically effective amount of a substantially pure LPS antagonist **isolated** from a gram negative bacterium that is defective in at least one of the msbB or htrB genes, or an analog or derivative thereof, wherein the LPS antagonist has reduced pyrogenicity relative to an LPS antagonist **isolated** from the wildtype bacterium, and (ii) a vaccine antigen, which is not **isolated** from the gram negative bacterium.

12. Document ID: US 6746870 B1

L29: Entry 12 of 16 File: USPT Jun 8, 2004

DOCUMENT-IDENTIFIER: US 6746870 B1
TITLE: DNA recombination in eukaryotic cells by the bacteriophage PHIC31 recombination system

Detailed Description Text (5):
The term "**isolated**", when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. In particular, an **isolated** gene is separated from open reading frames which flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

Detailed Description Text (6):
The term "naturally-occurring" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be **isolated** from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

Detailed Description Text (11):
"Promoter" refers to a region of DNA involved in binding the RNA polymerase to initiate transcription. An "**inducible promoter**" refers to a promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, **temperature**, pH, transcription factors and chemicals.

Detailed Description Text (38):
A promoter can be derived from a gene that is under investigation, or can be a heterologous promoter that is obtained from a different gene, or from a different species. Where direct expression of a gene in all **tissues** of a transgenic plant or other organism is desired, one can use a "constitutive" promoter, which is generally active under most environmental conditions and states of development or cell differentiation. Suitable constitutive promoters for use in plants include, for example, the cauliflower mosaic virus (CaMV) 35S transcription initiation region and region VI promoters, the 1'- or 2'-promoter derived from T-DNA of Agrobacterium tumefaciens, and other promoters active in plant cells that are known to those of skill in the art. Other suitable promoters include the full-length transcript promoter from Figwort mosaic virus, actin promoters, histone promoters, tubulin promoters, or the mannopine synthase promoter (MAS). Other constitutive plant promoters include various ubiquitin or polyubiquitin promoters derived from, inter alia, Arabidopsis (Sun and Callis, Plant J., 11(5):1017-1027 (1997)), the mas, Mac or DoubleMac promoters (described in U.S. Pat. No. 5,106,739 and by Comai et al., Plant Mol. Biol. 15:373-381 (1990)) and other transcription initiation regions from various plant genes known to those of skill in the art. Such genes include for example, ACT11 from Arabidopsis (Huang et al., Plant Mol. Biol. 33:125-139 (1996)), Cat3 from Arabidopsis (GenBank No. U43147, Zhong et al., Mol. Gen. Genet. 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from Brassica napus (Genbank No. X74782, Solocombe et al., Plant Physiol. 104:1167-1176 (1994)), GPc1 from maize (GenBank No. X15596, Martinez et al., J. Mol. Biol 208:551-565 (1989)), and Gpc2 from maize (GenBank No. U45855, Manjunath et al., Plant Mol. Biol. 33:97-112 (1997)). Useful promoters for plants also include those obtained from Ti- or Ri-plasmids, from plant cells, plant viruses or other hosts where the promoters are found to be functional in plants. Bacterial promoters that function in plants, and thus are suitable for use in the methods of the invention include the octopine synthetase promoter, the nopaline

synthase promoter, and the manopine synthetase promoter. Suitable endogenous plant promoters include the ribulose-1,6-biphosphate (RUBP) carboxylase small subunit (ssu) promoter, the (.alpha.-conglycinin promoter, the phaseolin promoter, the ADH promoter, and heat-shock promoters.

Detailed Description Text (40):
Alternatively, one can use a promoter that directs expression of a gene of interest in a specific **tissue** or is otherwise under more precise environmental or developmental control. Such **promoters are referred to here as "inducible" or "repressible" promoters**. Examples of environmental conditions that may effect transcription by **inducible promoters** include pathogen attack, anaerobic conditions, ethylene or the presence of light. Promoters under developmental control include promoters that initiate transcription only in certain **tissues**, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an **inducible promoter** may become fully or partially constitutive in certain locations. **Inducible promoters** are often used to control expression of the recombinase gene, thus allowing one to control the timing of the recombination reaction. Examples of **tissue**-specific plant promoters under developmental control include promoters that initiate transcription only in certain **tissues**, such as fruit, seeds, or flowers. The **tissue**-specific E8 promoter from tomato is particularly useful for directing gene expression so that a desired gene product is located in fruits. See, e.g., Lincoln et al. (1988) Proc. Nat'l. Acad. Sci. USA 84: 2793-2797; Deikman et al. (1988) EMBO J. 7: 3315-3320; Deikman et al. (1992) Plant Physiol. 100: 2013-2017. Other suitable promoters include those from genes encoding embryonic storage proteins. Examples of environmental conditions that may affect transcription by **inducible promoters** include anaerobic conditions, elevated **temperature**, or the presence of light. Additional organ-specific, **tissue**-specific and/or **inducible foreign promoters** are also known (see, e.g., references cited in Kuhlemeier et al (1987) Ann. Rev. Plant Physiol. 38:221), including those 1,5-ribulose bisphosphate carboxylase small subunit genes of Arabidopsis thaliana (the "ssu" **promoter), which are light-inducible** and active only in photosynthetic **tissue**, anther-specific promoters (EP 344029), and seed-specific promoters of, for example, Arabidopsis thaliana (Krebbers et al. (1988) Plant Physiol. 87:859). Exemplary green **tissue**-specific promoters include the maize phosphoenol pyruvate carboxylase (PEPC) promoter, small submit ribulose bis-carboxylase promoters (ssRUBISCO) and the chlorophyll a/b binding protein promoters. The promoter may also be a pith-specific promoter, such as the promoter **isolated** from a plant TrpA gene as described in International Publication No. WO93/07278.

Detailed Description Text (43):
The construction of polynucleotide constructs generally requires the use of vectors able to replicate in bacteria. A plethora of kits are commercially available for the purification of plasmids from bacteria. For their proper use, follow the manufacturer's instructions (see, for example, EasyPrepJ, FlexiPrepJ, both from Pharmacia Biotech; StrataCleanJ, from Stratagene; and, QIAexpress Expression System, Qiagen). The **isolated** and purified plasmids can then be further manipulated to produce other plasmids, used to transfect cells or incorporated into Agrobacterium tumefaciens to infect and transform plants. Where Agrobacterium is the means of transformation, shuttle vectors are constructed. Cloning in Streptomyces or Bacillus is also possible.

Detailed Description Text (48):
The polynucleotide constructs that include recombination sites and/or recombinase-encoding genes can be introduced into the target cells and/or organisms by any of the several means known to those of skill in the art. For instance, the DNA constructs can be introduced into plant cells, either in culture or in the organs of a plant by a variety of conventional techniques. For example, the DNA constructs can be introduced directly to plant cells using biolistic methods, such as DNA particle bombardment, or the DNA construct can be introduced using techniques such

as electroporation and microinjection of plant cell protoplasts. Particle-mediated transformation techniques (also known as "biolistics") are described in Klein et al., Nature, 327:70-73 (1987); Vasil, V. et al., Bio/Technol. 11:1553-1558 (1993); and Becker, D. et al., Plant J., 5:299-307 (1994). These methods involve penetration of cells by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface. The biolistic PDS-1000 Gene Gun (Biorad, Hercules, Calif.) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells. The process is applicable to a wide range of **tissues** and cells from organisms, including plants, bacteria, fungi, algae, intact animal **tissues**, **tissue** culture cells, and animal embryos. One can employ electronic pulse delivery, which is essentially a mild electroporation format for live **tissues** in animals and patients. Zhao, Advanced Drug Delivery Reviews 17:257-262 (1995).

Detailed Description Text (55):
Transformed plant cells, derived by any of the above transformation techniques, can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a **tissue** culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts **Isolation** and Culture, Handbook of Plant Cell Culture, pp. 124-176, Macmillian Publishing Company, New York (1983); and in Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, (1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar et al., J. **Tissue** Cult. Meth., 12:145 (1989); McGranahan et al., Plant Cell Rep., 8:512 (1990)), organs, or parts thereof. Such regeneration techniques are described generally in Klee et al., Ann. Rev. of Plant Phys., 38:467-486 (1987).

Detailed Description Text (56):
The methods are useful for producing transgenic and chimeric animals of most vertebrate species. Such species include, but are not limited to, nonhuman mammals, including rodents such as **mice** and rats, rabbits, ovines such as sheep and goats, porcines such as pigs, and bovines such as cattle and buffalo. Methods of obtaining transgenic animals are described in, for example, Puhler, A., Ed., Genetic Engineering of Animals, VCH Publ., 1993; Murphy and Carter, Eds., Transgenesis Techniques: Principles and Protocols (Methods in Molecular Biology, Vol. 18), 1993; and Pinkert, C A, Ed., Transgenic Animal Technology: A Laboratory Handbook, Academic Press, 1994. Transgenic fish having specific genetic modifications can also be made using the claimed methods. See, e.g., Iyengar et al. (1996) Transgenic Res. 5: 147-166 for general methods of making transgenic fish.

Detailed Description Text (57):
One method of obtaining a transgenic or chimeric animal having specific modifications in its genome is to contact fertilized oocytes with a vector that includes the polynucleotide of interest flanked by recombination sites. For some animals, such as **mice** fertilization is performed in vivo and fertilized ova are surgically removed. In other animals, particularly bovines, it is preferably to remove ova from live or slaughterhouse animals and fertilize the ova in vitro. See DeBoer et al., WO 91/08216. In vitro fertilization permits the modifications to be introduced into substantially synchronous cells. Fertilized oocytes are then cultured in vitro until a pre-implantation embryo is obtained containing about 16-150 cells. The 16-32 cell stage of an embryo is described as a morula. Pre-implantation embryos containing more than 32 cells are termed blastocysts. These embryos show the development of a blastocoel cavity, typically at the 64 cell stage. If desired, the presence of a desired exogenous polynucleotide in the embryo cells can be detected by methods known to those of skill in the art. Methods for culturing fertilized oocytes to the pre-implantation stage are described by Gordon et al. (1984) Methods Enzymol. 101: 414; Hogan et al. Manipulation of the **Mouse**

Embryo: A Laboratory Manual, C.S.H.L. N.Y. (1986) (**mouse** embryo); Hammer et al. (1985) Nature 315: 680 (rabbit and porcine embryos); Gandolfi et al. (1987) J. Reprod. Fert. 81: 23-28; Rexroad et al. (1988) J. Anim. Sci. 66: 947-953 (ovine embryos) and Eyestone et al. (1989) J. Reprod. Fert. 85: 715-720; Camous et al. (1984) J. Reprod. Fert. 72: 779-785; and Heyman et al. (1987) Theriogenology 27: 5968 (bovine embryos). Sometimes pre-implantation embryos are stored frozen for a period pending implantation. Pre-implantation embryos are transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is integrated. Chimeric mammals can be bred to form true germline transgenic animals.

Detailed Description Text (71):
The 84 bp .PHI.C31 attP site (abbreviated as PP'), **isolated** as an ApaI-SacI fragment from pHS282 (Thorpe & Smith (1998) Proc. Nat'l. Acad. Sci. USA 95:5505-5510) was cloned into the same sites of the S. pombe integrating vector pJK148 (Keeney & Boeke (1994) Genetics 136:849-856) to make pLT44. This plasmid was targeted to the S. pombe leu1-32 allele by lithium acetate mediated transformation with NdeI cut DNA. The recipient host FY527 (h.sup.- ade6-M216 his3-D1 leu1-32 ura4-D18), converted to Leu.sup.+ by homologous recombination with pLT44, was examined by Southern analysis. One Leu.sup.+ transformant, designated FY527attP, was found to contain a single copy of pLT44. Another transformant, designated FY527attP.times.2, harbors a tandem plasmid insertion.

Detailed Description Text (73):
The S. pombe ura4.sup.+ gene, excised from pTZura4 (S. Forsburg) on a 1.8 kb EcoRI-BamHI fragment, was inserted into pJK148 cut with the same enzymes to create pLT40. The .PHI.C31 attB site (abbreviated as BB'), **isolated** from pHS21 as a 500 bp BamHI-XbaI fragment, was ligated into pLT40 cut with those enzymes, creating pLT42. Most of the leu1 gene was removed from pLT42 by deleting a XhoI fragment to create pLT45. This removed all but 229 bp of leu1 from pLT45 and reduced its transformation efficiency to that of a plasmid without any leu1 homology. pLT50, which has a second attB site in the same orientation immediately on the other side of ura4, was constructed by first subcloning the attB BamHI-SacI fragment from pLT42 into pUC19, excising it with EcoRI and SalI, and subsequently inserting it into pLT45 cut with EcoRI and XhoI. The second attB site in the final construct was sequenced once on each strand and found to be identical to the first attB site.

Detailed Description Text (86):
Recombination between the pLT45-encoded .PHI.C31 attB element and the chromosomally situated attP sequence would incorporate the circular DNA into the leu1 locus as depicted in FIG. 1B. If this reaction occurs, XbaI-fractionated genomic DNA from the Ura.sup.+ transformants is probed with leu1 DNA, the 3 kb band will remain unchanged, while the 18 kb band will increase to .about.23 kb (FIG. 1C). Randomly selected Ura.sup.+ colonies were examined by hybridization analysis. Of eight **isolates** derived from experiments where .PHI.C31 integrase gene expression was derepressed by the omission of thiamine, seven showed the presence of this .about.23 kb band. This same size band hybridized to the ura4 probe. This contrasts with the lack of ura4 hybridization with the parental strain, as expected from its ura4-D18 deletion allele. One of these seven **isolates** showed additional bands hybridizing to both probes. This candidate appears to have a DNA rearrangement at the leu1 locus in addition to a site-specific recombination event. The leu1 rearrangement was probably catalyzed by the operative S. pombe homologous recombination system. The remaining **isolate** had not experienced a site-specific recombination event and appeared to have gained uracil prototrophy by recombination between pLT45 and pLT43. Of these eight **isolates**, half were selected as both Ura.sup.+ and His.sup.+, but no significant difference was found between this group and the group selected for Ura.sup.+ only.

Detailed Description Text (87):
From transformation experiments plated in the presence of vitamin B1, an equal

number of Ura.sup.+ transformants was examined by DNA hybridization. The thiamine-repressible Pnmt promoter is expected to limit integrase production, and thereby site-specific integration. Two of the eight Ura.sup.+ candidates **isolated** from this low frequency transformation showed a band of 23 kb hybridizing to leu1 and to the ura4 probe. However, since both probes detected an additional band, they do not represent correct integration events, and we grouped them as class b integrants. In the other six **isolates**, the hybridization patterns are difficult to interpret. In some of them, the 3 kb band was not detected by the leu1 probe, as though the locus has experienced some rearrangement. In many of them, the weak hybridization to ura4 suggests that the Ura.sup.+ phenotype may not be due to the stable maintenance of pLT45 in the genome.

Detailed Description Text (88):
To ascertain the proportion of transformants maintaining the integrase plasmid in the absence of selection, the blots were re-probed with the integrase gene sequence. Those selected as Ura.sup.+ His.sup.+ would be expected to maintain the plasmid, and did so, as the hybridization revealed. Five of the eight **isolates** selected as Ura.sup.+ without regard to the His phenotype also gave bands hybridizing to the integrase probe. To confirm that loss of int would not affect stable integration, another set of randomly chosen Ura.sup.+ cells were grown non-selectively for a number of generations and screened for His.sup.- progeny that have lost pLT43. The analysis of eight representative Ura.sup.+ His.sup.- clones showed that all had a single copy of pLT45 precisely integrated at the chromosome-situated attP site. The DNA of these integrants did not hybridize with the integrase probe. In contrast, the background frequency Ura.sup.+ clones derived by transformation of pLT45 alone gave the parental configuration of hybridizing bands at the leu1 locus and additional faint bands at 5 kb and 7 kb. These observations are consistent with either integration of pLT45 elsewhere in the genome, or maintenance of the plasmid in some cells despite the lack of a S. pombe replication origin.

Detailed Description Text (93):
Three Ura.sup.- His.sup.+ clones from each of the three cultures that had been transformed by pLT45 were analyzed by Southern blotting. One **isolate** had a DNA pattern consistent with stable integration of pLT45 into FY527attP. Therefore, in this clone, the Ura.sup.- phenotype was caused by a mutation that did not appreciably alter the restriction pattern, rather than by reversal of the site-specific recombination reaction. The second clone showed a Southern pattern characteristic of FY527attP lacking a pLT45 insertion, the third had a pattern consistent with a mixture of two types of cells, those like FY527attP without a pLT45 insertion, and those like the FY527attP progenitor strain FY527. The latter structure could arise from intrachromosomal homologous recombination between the leu repeats, reversing the insertion of pLT44 (FIG. 1A). If precise excision of the integrated plasmid DNA occurred in the latter two candidates, the attP site would be regenerated; this would be detectable with PCR. The size of the PCR product was that expected for an intact hybrid site, the presence of the hybrid site was confirmed by sequencing the PCR product. These observations are consistent with the idea that deletion of the ura4 gene occurred by some mechanism other than .PHI.C31-mediated excision.

Detailed Description Text (100):
The CHO cell line 51YT211 was transfected with the attP-containing plasmid pFY1, which included a selectable marker that confers zeocin resistance (FIG. 2). After being single colony purified twice, six zeocin resistant cell lines were **isolated**. Analysis by Southern DNA hybridization confirmed that each of the six cell lines had at least one molecule of pFY1 integrated into the genome.

Detailed Description Text (135):
If the two constructs were present in the same genome, the expression of int from the pWP24 bearing chromosome would be expected to produce functional .PHI.C31

integrase to catalyze the recombination between attB and attP sites situated on the pWP29-bearing chromosome. The recombination event would be expected to delete the npt gene from the pWP29 construct and fuse 35S to gus. The resulting configuration would be 35S-attR-gus, where attR is a hybrid site formed by the recombination between attP and attB, also designated as PB' (FIG. 4). The deletion of npt brings gus under the transcription of 35S and would be expected to yield plants with GUS enzyme activity. This activity can be detected through histochemical staining of the plant **tissue**.

Detailed Description Text (140):
The intensity of staining varied depending on the combination of lines used as parental lines. Those with progeny with a greater proportion of the **tissue** staining blue indicate that the recombination event was more efficient. Conversely, those yielding progeny with less uniform staining indicate that the recombination event was less efficient. This variation among the different progeny pools is probably due to effects caused by the position of integration of the transgenes. Of the two integrase lines, 24.4 appears more efficient in promoting site-specific recombination. This is probably due to a higher level of int gene expression. Staining patterns produced by crossing 24.4 to 29.4 and 29.19 are consistent with the experimental design that int promoted site-specific recombination of attB and attP results in the activation of gus gene activity.

Other Reference Publication (61):
Becker et al., "Fertile transgenic wheat from microprojectile bombardment of scutellar **tissue**," The Plant Journal, 5(2):299-307 (1994).

Other Reference Publication (98):
Nehra et al., "Self-fertile transgenic wheat plants regenerated from **isolated** scutellar **tissues** following microprojectile bombardment with tow distinct gene constructs," The Plant Journal, 5(2):285-297 (1994).

Other Reference Publication (108):
Schmidt et al., "Illegitimate Cre-dependent chromosome rearrangements in transgenic **mouse** spermatids," PNAS, 97:13702-7 (2000).

CLAIMS:

6. The method of claim 5, wherein the recombinase polypeptide is selected from the group consisting of a bacteriophage .PHI.C31 integrase, a coliphage P4 recombinase, a **Listeria** phage recombinase, a bacteriophage R4 Sre recombinase, a CisA recombinase, an XisF recombinase, and a transposon Tn4451 TnpX recombinase.

13. Document ID: US 6537558 B2

L29: Entry 13 of 16 File: USPT Mar 25, 2003

DOCUMENT-IDENTIFIER: US 6537558 B2
TITLE: Methods of producing and using virulence attenuated poxR mutant bacteria

Brief Summary Text (3):
The poxA gene (Van Dyk et al., J. Bacteriology169(10):4540-4546 (1987)), is a

regulatory gene affecting expression of pyruvate oxidase (Chang and Cronan, J. Bacteriology 151(3): 1279-1289 (1982)). The poxA gene of E. coli is located at min 94. Enzymological and immunological data indicate that mutations in poxa have an 8 to 10-fold decrease in pyruvate oxidase levels (Chang and Cronan (1982); Chang and Cronan, J. Bacteriol. 154:756-762 (1983)). It has also been reported that poxA mutants grow more slowly than the isogenic wild-type in both minimal and rich media, while poxB mutants exhibit normal growth. Van Dyk and LaRossa, J. Bacteriol. 165(2):386-392 (1986), **isolated** 15 mutant Salmonella typhimurium strains sensitive to the herbicide sulfometuron methyl (SM) [N-[(4,6-dimethylpyrimidin-2-yl) aminocarbonyl]-2-methoxycarbonyl-benzenesu lfonamide], following Tn10 mutagenesis. Among these SM-hypersensitive mutations, a poxA mutation was identified and mapped to the 94 min region of S. typhimurium genetic map (Van Dyk et al. (1987)), a location analogous to that of poxA in E. coli. The S. typhimurium poxA mutant, similarly to the E. coli poxA mutant, had reduced pyruvate oxidase activity and reduced growth rates (Van Dyk et al. (1987)). Furthermore, the E. coli and S. typhimurium poxA mutants shared several additional phenotypes including hypersensitivity to SM, to .alpha.-ketobutyrate, and to a wide range of bacterial growth inhibitors, such as antibiotics, amino acid analogs and dyes (Van Dyk et al. (1987)).

Brief Summary Text (4):
The immune system of animals is especially suited to reacting to and eliminating microorganisms which infect the animal. The sustained presence of the full range of antigens expressed by infecting microorganisms provide a stimulating target for the immune system. It is likely that these characteristics lead to the superior efficacy, on average, of vaccines using live attenuated virus. For similar reasons, live bacterial vaccines have been developed that express a desired antigen and colonize the intestinal tract of animals (Curtiss et al., Curr. Topics Micro. Immun. 146:35-49 (1989); Curtiss, Attenuated Salmonella Strains as Live Vectors for the Expression of Foreign Antigens, in New Generation Vaccines (Woodrow and Levine, eds., Marcel Dekker, New York, 1990) pages 161-188; Schodel, Infection 20(1): 1-8 (1992); Cardenas and Clements, Clinical Micro. Rev. 5(3):328-342 (1992)). Most work to date has used avirulent Salmonella typhimuriumstrains synthesizing various foreign antigens for immunization of **mice**, chickens and pigs. Several avirulent S. typhi vectors have been evaluated in human volunteers (Tacket et al., Infect. Immun.60:536-541 (1992)) and several phase I clinical trials with recombinant avirulent S. typhi strains are in progress in the U.S. and Europe. An important safety advantage of the live attenuated bacterial vaccine vectors as compared to the use of viral vector based vaccines is the ability to treat an immunized patient with oral ciprofloxacin or amoxicillin, should an adverse reaction occur.

Brief Summary Text (9):
It is another object of the present invention to provide an **isolated** nucleic acid molecule containing or encoding the poxR gene.

Drawing Description Text (2):
FIG. 1 is a graph of the number of poxR mutant S. typhimurium UK-1 cells colonizing various organs (measured in colony forming units per gram of organ) in day-old chicks. Chicks were orally inoculated with 1.times.10.sup.8 cfu of poxR401::Tn10 strain MGN-791s at day of hatch. All birds survived and six days post-infection, the colonization levels in the **spleen**, **liver**, bursa and cecum were determined.

Drawing Description Text (3):
FIG. 2 is a graph of the number of poxR mutant S. typhimurium cells colonizing various organs (measured in mean colony forming units per organ) in BALB/c **mice**. Each time point consisted of group of three female BALB/c **mice** six weeks-old. **Mice** were inoculated orally (p.o.) with 2.6.times.10.sup.9 cfu at day 1. Three and seven days post-inoculation, **mice** were humanely euthanized and **spleens** (SP), mesenteric lymph nodes (MLN), and Peyer's patches (PP) were removed. The organs were disrupted in one milliliter of buffer saline and 0.1 ml of appropriate dilutions were plated

in triplicate on MacConkey lactose plates.

Drawing Description Text (4):
FIGS. 3A, 3B, and 3C are graphs of the immune responses (measured as optical density at 405 nm of ELISA assays) of **mice** immunized with various amounts of S. typhimurium poxR mutant. The optical density at 405 nm in the ELISA assays is a measure of the level of immunoreactive antibodies present. FIG. 3A graphs the IgA response. FIG. 3B graphs the IgM response. FIG. 3C graphs the IgG response. Each treatment group consisted of five female BALB/c **mice** six weeks-old. **Mice** were inoculated intra peritoneally (i.p.) with 2.6.times.10.sup.4 cfu, and orally (p.o.) with 2.6.times.10.sup.7, 2.6.times.10.sup.8, and 2.6.times.10.sup.9 cfu at day 1. No booster immunization was performed. At day 28 post-immunization, sera were collected from immunized and non immunized control **mice** and subjected to an ELISA assay using purified Salmonella LPS as coating antigen. The graphs represent the mean absorbance at 405 nm (OD) for five **mice**. For individual **mice**, the serum was considered positive when the OD was greater than the mean OD of control **mice** plus two times the standard deviation of the same control sera. For the IgA response, 5/5 were positive in the i.p. group, 3/5 in the p.o. 2.6.times.10.sup.7 cfu group; 5/5 in the p.o. 2.6.times.10.sup.8 cfu group; 3/5 in the p.o. 2.6.times.10.sup.9 cfu group. For the IgM response, 3/5 were positive in the i.p. group; 2/5 in the p.o. 2.6.times.10.sup.7 cfu group; 2/5 in the p.o. 2.6.times.10.sup.8 cfu group; 2/5 in the p.o. 2.6.times.10.sup.9 cfu group. For the IgG response, 5/5 were positive in the i.p. group; 4/5 in the p.o. 2.6.times.10.sup.7 cfu group; 5/5 in the p.o. 2.6.times.10.sup.8 cfu group; 3/5 in the p.o. 2.6.times.10.sup.9 cfu group.

Detailed Description Text (20):
It is preferred that bacterial cells having a poxR mutation also contain other features which attenuate their virulence and increase their immunogenicity. For example, strains of different Salmonella serotypes can be rendered avirulent by methods known to those skilled in the art, for example, 1) by introducing mutations that impose a requirement for aromatic amino acids and vitamins derived from precursors in this pathway (Stocker et al., Dev. Biol. Stand. 53:47-54 (1983), Hoiseth et al., Nature 291(5812):238-239 (1981)), 2) by mutating genes for global regulators such as cya and crp (Curtiss and Kelly, Infect. Imm. 55:3035 (1987)), phoP (Miller et al., Proc. Natl. Acad. Sci. USA 86:5054-8 (1989), Galan and Curtiss, Microb. Pathogen. 6:433-443 (1989)), and ompR (Dorman et al., Infect. Immun. 57:2136-40 (1989)), 3) by mutating genes for lipopolysaccharide (LPS) synthesis, such as by galE (Germanier and Furer, Infect. Immun. 4:663-73 (1971), Germanier and Furer, J. Infect. Dis. 131:553-8 (1975)), although this alone may be insufficient (Hone et al., Infect. Immun.56:1326-1333 (1988)), pmi (Collins et al., Infect. Immun. 59:1079-1085 (1991)), 4) by mutating genes needed for colonization of deep **tissues**, such as cdt (Kelly et al., Infect. Immun. 60:4881-4890 (1992); Curtiss et al., Devel. Biol. Stand. 82:23-33 (1994)), or 5) by preventing expression of genes for proteases required at high temperature, such as htrA (Johnson et al., Mol. Microbiol. 5:401-407 (1991). Strains possessing mutations in phoQ (Miller et al., Proc. Natl. Acad. Sci. USA 86:5054 (1989)) have the same phenotype as mutations in phoP. Strains with mutations in either phoP or phoQ are referred to herein collectively as phoP mutants. It is preferred that mutations in the above described genes be introduced as deletions since this will preclude reversion mutations and enhance the safety of the strains containing them. Subsequent to the discovery that Salmonella strains with mutations in the genes described above are avirulent and immunogenic, it was observed that many of these strains exhibited, after oral administration, nearly wild-type abilities to invade and persist in the GALT and to colonize other lymphoid **tissues** such as mesenteric lymph nodes, **liver, and spleen**, but without causing disease symptoms. As a consequence, these attenuated strains are capable of stimulating strong mucosal, systemic and cellular immune responses in irimmunized animal hosts that confer protective immunity to challenge with virulent wild-type Salmonella strains.

Detailed Description Text (27):
Virulence of the disclosed bacterial cells can be assessed by, for example, using the chick model described in Example 6, or the **mouse** model described in Examples 7 and 8. As used herein, attenuated virulence refers to a reduction in virulence of bacteria relative to a reference bacteria of the same type. It is preferred that the reference bacteria is a wild-type bacteria. Most preferably, the reference bacteria is the parent strain from which a poxR mutant was generated. Avirulent does not mean that a bacterial cell of that genus or species can not ever function as a pathogen, but that the particular bacterial cell being used is avirulent with respect to the particular animal being treated. The bacterial cell may belong to a genus or even a species that is normally pathogenic but must belong to a strain that is avirulent. By pathogenic is meant capable of causing disease or impairing normal physiological functioning. Avirulent strains are incapable of inducing a full suite of symptoms of the disease that is normally associated with its virulent pathogenic counterpart.

Detailed Description Text (31):
The disclosed bacterial cells having a poxR mutation can be used to induce an immune response to related bacterial cells (since the disclosed bacterial cells will present their native antigens to a host animal when administered), or, preferably, the disclosed cells can be used as an antigen delivery system. As used herein, the terms antigen delivery system and antigen delivery bacteria refer to bacterial cells that produce an antigen or that harbor a vector encoding an antigen. In one preferred embodiment of an antigen delivery system using the disclosed bacterial cells, pathogenic bacterial cells that attach to, invade and persist in the gut-associated lymphoid **tissue** (GALT) or bronchial-associated lymphoid **tissue** (BALT), and that have a poxR mutation are used as a carrier of a gene product which is used for stimulating immune responses against a pathogen or allergen.

Detailed Description Text (33):
Shigella or an enteroinvasive E. coli having a poxR mutation can be useful in antigen delivery systems since invasion into colonic mucosa could stimulate lymphoid **tissues** adjacent to the colon, so as to stimulate a strong mucosal immune response in the reproductive tract. Rectal immunization can be effective because of anatomical features such as the proximity of lymph nodes and lymphatics to the colon.

Detailed Description Text (34):
Antigens. Live bacteria having a poxR mutation can be used to deliver any product that can be expressed in the bacteria. Preferred expression products for this purpose are antigens. For example, antigens can be from bacterial, viral, mycotic and parasitic pathogens, to protect against bacterial, viral, mycotic, and parasitic infections, respectively; gametes, provided they are gamete specific, to block fertilization; and **tumor** antigens, to halt cancers. It is specifically contemplated that antigens from organisms newly identified or newly associated with a disease or pathogenic condition, or new or emerging pathogens of animals or humans, including those now known or identified in the future, can be used with the disclosed bacterial cells and methods. Antigens for use in the disclosed bacterial cells are not limited to those from pathogenic organisms. The selection and recombinant expression of antigens has been previously described by Schodel (1992) and Curtiss (1990). It is preferred that a gene for expression in the disclosed bacterial cells be operably linked to a promoter of any gene of the type III secretion system, preferably a promoter of a sip (ssp) gene, a yop gene, a ipa gene, or a hrp gene. It is also preferred that an expression product for the disclosed bacterial cells be expressed as a fusion to a Sip (Ssp) protein, a Yop protein, a Ipa protein, or a Hrp protein. Such a fusion is preferably expressed using the natural promoter with which the protein is expressed. Immunogenicity of the bacterial cells can be augmented and/or modulated by constructing strains that also express genes for cytokines, adjuvants, and other immunomodulators.

Detailed Description Text (40):
Antigen Delivery Compositions. A preferred use of the disclosed bacteria is as vaccines for stimulating an immune response to the delivered antigens. Oral immunization in a suitable animal host with live recombinant Salmonella having a poxR mutation leads to colonization of the gut-associated lymphoid **tissue** (GALT) or Peyer's patches, which leads to the induction of a generalized mucosal immune response to both Salmonella antigens and any foreign antigens synthesized by the recombinant Salmonella (Curtiss et al., Adv. Exp. Med. Biol. 251:33-47 (1989)). Further penetration of the bacteria into the mesenteric lymph nodes, **liver and spleen** augments the induction of systemic and cellular immune responses directed against Salmonella antigens and the foreign antigens made by the recombinant Salmonella (Doggett and Curtiss (1992)). Thus the use of recombinant Salmonella for oral immunization stimulates all three branches of the immune system, particularly important when immunizing against infectious disease agents which colonize on and/or invade through mucosal surfaces.

Detailed Description Text (68):
It has been reported that S. typhimurium mutants with reduced virulence also show sensitivity to antimicrobial cationic peptides such as defensin and protamine (Groissman et al., Proc. Natl. Acad. Sci. USA 89:11939-11943 (1992)). MGN-1036s failed to grow in presence of 1.25 mg/ml of protamine sulfate, while the isogenic wild-type exhibited normal growth at that concentration. This phenotype was complementable by pMEG-274. The defined deletion strain MGN-1036s was also found to be attenuated for virulence in **mice**. MGN-1036s was characterized biochemically using API strips. The results showed no biochemical difference between wild-type and poxR mutants. The growth rate of the defined deletion strain MGN-1036s was identical to that of the previous poxR mutants MGN-791s and MGN-816s. Taken together, the poxR defined mutant showed a phenotype identical to the transposon generated deletion strain and the insertion strain.

Detailed Description Text (80):
Day-old chicks are very sensitive to S. typhimurium UK-1 (Hassan and Curtiss, Infect. Immun. 62(12):5519-5527 (1994)). The 50% lethal dose (LD.sub.50) of wild-type bacteria in day of hatch chicks in 3.times.10.sup.3 cfu. Therefore, infant chicks are excellent models for S. typhimurium infection. It had been previously determined that the mean time to death of day-old chicks infected with 1.times.108 cfu of wild-type S. typhimurium UK-1 was 3 days. In order to determine whether the poxR mutation has any effect on virulence, five specific pathogen free white Leghorn chicken were inoculated by oral gavage with 1.times.10.sup.8 cfu of strain MGN-791s (see Table 1) at day of hatch. Per oral inoculation (p.o.) of day-old chicks with S. typhimurium strains MGN-791s was performed according to Hassan and Curtiss. Under these conditions, all birds survived six days post-infection. To determine whether the survival of birds was due to the inability of the bacteria to disseminate in target organs, the colonization levels in the **spleen, liver,** bursa and cecum were determined six days post-infection (FIG. 1). The results show that the poxR mutant strain MGN 791s was able to colonize deep **tissues**. The survival of birds to 10,000 times the LD.sub.50 dose is an indication of attenuation of virulence due to poxR mutation.

Detailed Description Text (82):
Attenuation of Virulence and Colonization in **Mice** by poxR Mutants

Detailed Description Text (83):
To confirm the role of poxR mutation in virulence, the 50% lethal dose (LD.sub.50) of S. typhimurium strain MGN-816s (see Table 1) was determined in **mice**. Treatment groups of six week-old female BALB/c **mice** were inoculated at day 1 either intra peritoneally (i.p.), or orally (p.o.) with the doses indicated in Table 2. Per oral inoculation (p.o.) and intraperitoneal (i.p.) inoculations of 6 week old female BALB/c **mice** with the different S. typhimurium strains were carried out as

previously described (Galan and Curtiss (1989)). A non inoculated control group was included. **Mice** which survived immunization were challenged 35 days post-immunization by oral inoculation with 7.5.times.10.sup.8 cfu of wild-type UK-1 strain .sub.x 3761. Control **mice** were challenged by oral inoculation with 1.5.times.10.sup.4 cfu of wild type UK-1 strain .sub.x 3761 (the equivalent of LD.sub.50). Three control **mice** died within 11 days post-challenge. The 2 remaining control **mice** were moribund and looked scruffy. They were euthanized and scored as dead from infection.

Detailed Description Text (84):
Three and seven days post-inoculation, three **mice** were humanely euthanized from the 2.6.times.10.sup.9 cfu treatment group, and the colonization levels in the **spleen**, mesenteric lymph nodes and Peyer's patches were determined (FIG. 2). The results show greater than 10.sup.4 cfu in both **spleen** and mesenteric lymph nodes, indicating that poxR mutant derivatives of S. typhimurium UK-1 are capable of colonizing deep **tissues in mice** following oral inoculation.

Detailed Description Text (85):
Following immunization, **mice** were monitored daily for death. All **mice** in the i.p. 2.6.times.10.sup.5 cfu group died within 13 days post-infection. However, all **mice** from other treatment groups survived after 12 days post-inoculation (Table 2). The LD.sub.50 of wild-type S. typhimurium UK-1 strain .sub.x 3761 was approximately 10 cfu for i.p., and 10.sup.4 cfu for p.o. in six week-old female BALB/c **mice**. These results indicate that poxR mutants of S. typhimurium are avirulent in both i.p. and p.o. since **mice** inoculated with poxR mutant survived approximately 1,000 times the i.p. LD.sub.50 of wild-type and 100,000 times the p.o. LD.sub.50 of wild-type. The attenuation of virulence was not the result of the inability of the strain to colonize deep **tissues** (see above).

Detailed Description Text (88):
Six week old female BALB/c **mice** were immunized with single doses as indicated in Table 2. At day 28 post-immunization, sera were collected by retro orbital eye bleeding from immunized and non-immunized control **mice**. The humoral immune response of **mice** to S. typhimurium poxR mutant was measured by ELISA using purified LPS as coating antigen, since the O-antigen is the immunodominant antigen in Salmonella. The humoral response to S. typhimurium poxR mutants was assayed by either ELISA, or Western blot (immunoblot) of whole cell lysates proteins separated by SDS-PAGE, transferred to nitrocellulose, and detected using polyclonal antisera from immunized **mice**, followed by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, Ill.). The results are shown in FIG. 3.

Detailed Description Text (89):
As expected, non-immunized control **mice** did not raise an immune response to Salmonella. However, **mice** immunized i.p. and p.o. raised strong IgA, IgM and IgG responses, indicating that poxR mutant derivatives of S. typhimurium induced a strong humoral immune response in **mice**. The humoral immune response measured in **mice** treated with 2.6.times.10.sup.9 cfu p.o. appeared to be suppressed.

Detailed Description Text (90):
It was then determined whether the immune responses induced by S. typhimurium poxR mutants could protect **mice** against lethal wild-type challenge. Day 35 post-immunization, both i.p. and p.o. immunized **mice** were challenged by oral inoculation with 7.5.times.10.sup.8 cfu of wild-type S. typhimurium UK-1 strain .sub.x 3761. The non-immunized control **mice** were challenged p.o. with 1.5.times.10.sup.4 cfu of wild-type, the equivalent of 1.5.times.LD.sub.50, to assess the virulence of the challenge strain. The experiment was terminated 16 days post-challenge and survivors following challenge were scored (Table 2). In the control treatment group, 5 of the 5 **mice** died by day 15 post-challenge. In immunized treatment groups, all **mice** survived approximately 10,000 times the LD.sub.50 of wild-type challenge after a single immunization regimen. Taken together, these results

demonstrate that poxR mutants are attenuated in **mice** and capable of inducing protective response against wild-type lethal challenge.

Detailed Description Paragraph Table (2):
TABLE 2 Survivors Survivors Route of Dose (cfu) of following following immunization bacteria immunization challenge Control -- -- 0/5 p.o. 2.6 .times. 10.sup.7 5/5 5/5 p.o. 2.6 .times. 10.sup.8 5/5 5/5 p.o. 2.6 .times. 10.sup.9 .sup. 5/5.sup.a .sup. 4/4.sup.b i.p. 2.6 .times. 10.sup.4 5/5 5/5 i.p. 2.6 .times. 10.sup.5 0/5 -- .sup.a Eleven **mice** were immunized and three **mice** were euthanized on days 3 and 7 to determine the level of colonization in the **spleen**, mesenteric lymph nodes, and Peyer's patches. .sup.b One **mouse** in this group died during serum collection on day 28 post-infection.

Other Reference Publication (8):
Sory et al., "Expression of the Eukaryotic Trypanosoma cruzi CRA Gene in Yersinia enterocolitica and Induction of an Immune Response against CRA in **Mice**" Infect. Immun. 60(9):3830-3836 (Sep. 1992).

Other Reference Publication (27):
Degryse, "**Stability** of a host-vector system based on complementation of an essential gene in Escherichia coli", J Biotechnol, 18:(1-2):29-39 (1991).

Other Reference Publication (37):
Ferrari, et al., "**Isolation** Of An Alanine Racemase Gene From Bacillus Subtilis And Its Use For Plasmid Maintenance in B. Subtilis", Bio/Technology, 3:1003-1007 (1985).

Other Reference Publication (51):
Germanier & Furer, "**Isolation** And Characterization Of Gal E Mutant Ty 21a Of Salmonella typhi: A Candidate Strain For A Live, Oral Typhoid Vaccine", J. Infect. Dis., 131:553-8 (1975).

Other Reference Publication (123):
Qoronfleh, et al., "Identification And Characterizaton Of Novel Low-**Temperature-Inducible Promoters** Of Escherichia coli", J. Bacteriol., 174:7902-7909 (1992).

Other Reference Publication (150):
Schweder, et al., "An Expression Vector System Providing Plasmid **stability** and Conditional Suicide of Plasmid-Containing Cells," Appl. Microbiol. Biotechnol., 38:91-93 (1992).

Other Reference Publication (158):
Stocker, et al., "Aromatic-Dependent <Salmonella Sp.> As Live Vaccine in **Mice** and Calves", Dev. Biol. Stand., 53:47-54 (1983).

Other Reference Publication (167):
Tinge & Curtiss, "**Isolation** of the Replication and Partitioning Regions of the Salmonella Typhimurium Virulence Plasmid and Stabilization of Heterologous Replicons," Journal of Bacteriology 172:5266-5277 (1990).

Other Reference Publication (193):
Collins, et al., "Mutations at rfc or pmi Attenuate Salmonella typhimurium Virulence for **Mice**", Infect. Immun., 59:1079-1085 (1991).

Other Reference Publication (196):
Curtiss & Tinge, "Recombinant Avirulent Salmonella Vaccines and Prospects for an Antifertility Vaccine," Local Immunicty in Reproductive Tract **Tissues**, pp. 459-476 (Oxford University Press, Griffin & Johnson, eds., 1993).

CLAIMS:

20. The method of claim 1 wherein the bacterial cells are in a genus selected from the group consisting of Salmonella, Shigella, Escherichia, Enterobacter, Serratia, Proteus, Yersinia, Citrobacter, Edwardsiella, Providencia, Klebsiella, Hafnia, Ewingella, Kluyvera, Morganella, Planococcus, Stomatococcus, Micrococcus, Staphylococcus, Vibrio, Aeromonas, Plessiomonas, Haemophilus, Actinobacillus, Pasteurella, Mycoplasma, Ureaplasma, Rickettsia, Coxiella, Rochalimaea, Ehrlichia, Streptococcus, Enterococcus, Aerococcus, Gemella, Lactococcus, Leuconostoc, Pediococcus, Bacillus, Corynebacterium, Arcanobacterium, Actinomyces, Rhodococcus, Listeria, Erysipelothrix, Gardnerella, Neisseria, Camylobacter, Arcobacter, Wolinella, Heliobacter, Achomobacter, Acinetobacter, Agrobacterium, Alcaligenes, Chryseomonas, Comamonas, Eikenella, Flavimonas, Flavobacterium, Moraxella, Oligella, Pseudomonas, Skewanella, Weeksella, Xanthomonas, Bordetella, Franciesella, Brucella, Legionella, Afipia, Bartonella, Calymmatobacterium, Cardiobacterium, Streptobacillus, Spirillum, Peptostreptococcus, Peptococcus, Sarcinia, Coprococcus, Ruminococcus, Propionibacterium, Mobiluncus, Bifidobacterium, Eubacterium, Lactobacillus, Rothia, Clostridium, Bacteroides, Porphyromonas, Prevotella, Fusobacterium, Bilophila, Leptotrichia, Wolinella, Acidaminococcus, Megasphaera, Veilonella, Norcardia, Actinomadura, Norcardiopsis, Streptomyces, Micropolysporas, Thermoactinomycetes, Mycobacterium, Treponema, Borrelia, Leptospira, and Chlamydiae.

14. Document ID: US 6500419 B1

L29: Entry 14 of 16 File: USPT Dec 31, 2002

DOCUMENT-IDENTIFIER: US 6500419 B1
TITLE: Method for introducing and expressing RNA in animal cells

Brief Summary Text (4):
Another new class of vaccines are bacterial vector vaccines (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, N.Y., pages 161-188 and 269-288 (1989); and Mims et al, In: Medical Microbiology, Eds., Mosby-Year Book Europe Ltd., London (1993)). These vaccines can enter the host, either orally, intranasally or parenterally. Once gaining access to the host, the bacterial vector vaccines express an engineered prokaryotic expression cassette contained therein that encodes a foreign antigen(s). Foreign antigens can be any protein (or part of a protein) or combination thereof from a bacterial, viral, or parasitic pathogen that has vaccine properties (New Generation Vaccines: The Molecular Approach, supra; Vaccines and Immunotherapy, supra; Hilleman, Dev. Biol. Stand., 82:3-20 (1994); Formal et al, Infect. Immun. 34:746-751 (1981); Gonzalez et al, J. Infect. Dis., 169:927-931 (1994); Stevenson et al, FEMS Lett., 28:317-320 (1985); Aggarwal et al, J. Exp. Med., 172:1083-1090 (1990); Hone et al, Microbial. Path., 5:407-418 (1988); Flynn et al, Mol. Microbiol., 4:2111-2118 (1990); Walker et al, Infect. Immun., 60:4260-4268 (1992); Cardenas et al, Vacc., 11:126-135 (1993); Curtiss et al, Dev. Biol. Stand., 82:23-33 (1994); Simonet et al, Infect. Immun., 62:863-867 (1994); Charbit et al, Vacc., 11:1221-1228 (1993); Turner et al, Infect. Immun., 61:5374-5380 (1993); Schodel et al, Infect. Immun., 62:1669-1676 (1994); Schodel et al, J. Immunol., 145:4317-4321 (1990); Stabel et al, Infect. Immun., 59:2941-2947 (1991); Brown, J. Infect. Dis., 155:86-92 (1987); Doggett et al, Infect. Immun., 61:1859-1866 (1993); Brett et al, Immunol., 80:306-312 (1993);

Yang et al, J. Immunol., 145:2281-2285 (1990); Gao et al, Infect. Immun., 60:3780-3789 (1992); and Chatfield et al, Bio/Technology, 10:888-892 (1992)). Delivery of the foreign antigen to the host **tissue** using bacterial vector vaccines results in host immune responses against the foreign antigen, which provide protection against the pathogen from which the foreign antigen originates (Mims, The Pathogenesis of Infectious Disease, Academic Press, London (1987); and New Generation Vaccines: The Molecular Approach, supra).

Brief Summary Text (8):
The delivery of endogenous and foreign genes to animal **tissue** for gene therapy has shown significant promise in experimental animals and volunteers (Nabel, Circulation, 91:541-548 (1995); Coovert et al, Curr. Opin. Neuro., 7:463-470 (1994); Foa, Bill. Clin. Haemat., 7:421-434 (1994); Bowers et al, J. Am. Diet. Assoc., 95:53-59 (1995); Perales et al, Eur. J. Biochem., 226:255-266 (1994); Danko et al, Vacc., 12:1499-1502 (1994); Conry et al, Canc. Res., 54:1164-1168 (1994); and Smith, J. Hemat., 1:155-166 (1992)).

Brief Summary Text (9):
From the onset nucleic acid vaccine studies focused on the use of DNA vaccines (Tang et al. (1992) supra; Fynan et al. (1993) supra; Donnelly et al. (1995) supra; Wang et al. (1993) supra; Davis et al. (1993) supra; Ulmer et al. (1993) supra; Robinson et al. (1993) supra; Eisenbraun et al. (1993) supra; Wang et al. (1994) supra; Coney et al. (1994) supra; Sedegah et al. (1994) supra; Raz et al. (1994) supra; Xiang et al. (1994) supra). More recently the use of RNA vaccines has been proposed as an alternative approach to the injection of DNA based nucleic vaccines (Zhou, X. et al. (1994) Vaccine 12:1510; Conry, R. M. et al. (1995) Cancer Res 55:1397). In support, "naked" RNA vaccines have proven modestly immunogenic in **mice** (Zhou et al. (1994) supra; Conry et al. (1995) supra). An RNA vaccine based on a recombinant Semliki Forrest Virus that expressed the SIV-PBj14 Env gene engendered protection against SIV-PBj14 (Mossman, S. P. et al. (1997) Journal of Virology 70:1953).

Brief Summary Text (11):
Thus, it is desirable to have an efficient method of delivering RNA to eukaryotic cells, such as mammalian cells, such that, the RNA can be expressed in the eukaryotic cell. Furthermore, it is also desirable to have a system permitting efficient delivery of RNA molecules to mucosal **tissue** in addition to permitting parenteral delivery of RNA molecules.

Brief Summary Text (13):
The invention provides a system for delivery of RNA molecules to eukaryotic cells, e.g., cells of mucosal **tissue**. The invention is based at least in part on the discovery that bacteria which are capable of invading eukaryotic cells can deliver RNA molecules to eukaryotic cells and **tissues**, and where appropriate, the RNA can be translated if the RNA contains the appropriate regulatory elements.

Brief Summary Text (14):
Accordingly, in one embodiment, the invention provides an **isolated** bacterium comprising a DNA which is transcribed into a messenger RNA molecule in the bacterium, wherein the RNA is capable of being translated in a eukaryotic cell, or is an antisense RNA or a catalytic RNA. The DNA can be heterologous with respect to the bacterium. The DNA can be operably linked to a prokaryotic promoter, e.g., the E. coli NirB promoter. Alternatively, the DNA can be operably linked to a first promoter, and the bacterium further comprises a gene encoding a polymerase, which is capable of mediating transcription from the first promoter, wherein the gene encoding the polymerase is operably linked to a second promoter. In a preferred embodiment the second promoter is a prokaryotic promoter. In a preferred embodiment, the polymerase is a bacteriophage polymerase, e.g., T7 polymerase, and the first promoter is a bacteriophage promoter, e.g., T7 promoter. The DNA which is capable of being transcribed into said RNA and the gene encoding a polymerase can

be located on one or more plasmids. However, in a preferred embodiment, the DNA is located on the bacterial chromosome.

Brief Summary Text (15):
In a preferred embodiment, the RNA can be translated in a eukaryotic cell. For allowing efficient translation in a eukaryotic cell, the RNA preferably comprises a Cap Independent Translation Enhancer (CITE) sequence. The RNA can further comprise additional regulatory elements, which can, e.g., affect the **stability** of the RNA in the eukaryotic cell, e.g., polyA tail. The RNA can encode one polypeptide. Alternatively, the RNA can be polycistronic and encode more than one polypeptide. The polypeptide can be, e.g., a vaccine antigen or an immunoregulatory molecule. The polypeptide can further be an endogenous or a foreign polypeptide. Foreign polypeptides include prokaryotic, e.g., bacterial, or viral polypeptides.

Brief Summary Text (17):
In another embodiment, the invention provides an **isolated** bacterium comprising an RNA which is capable of being translated in a eukaryotic cell, or is an antisense RNA, or a catalytic RNA. In a preferred embodiment, the RNA is transcribed in the bacterium, e.g., from introduced DNA. In another embodiment, the RNA is introduced into the bacterium by, e.g., electroporation. The RNA can be heterologous with respect to the bacterium.

Brief Summary Text (19):
A preferred invasive bacterium is Shigella, which is naturally invasive vis a vis vertebrate cells. At least one advantage of Shigella RNA vaccine vectors is their tropism for lymphoid **tissue** in the colonic mucosal surface. In addition, the primary site of Shigella replication is believed to be within dendritic cells and macrophages, which are commonly found at the basal lateral surface of M cells in mucosal lymphoid **tissues**. Thus, Shigella vectors provide a means to express antigens in these professional antigen presenting cells and thereby induce an immune response, e.g., a vaccine antigen.

Brief Summary Text (23):
The invention also provides **isolated** DNA operably linked to a prokaryotic promoter, wherein the DNA encodes RNA which is capable of being translated in a eukaryotic cell, or is an antisense RNA or a catalytic RNA, e.g., ribozyme. Preferred prokaryotic promoters are the E. coli llp promoter and NirB promoter.

Brief Summary Text (26):
The eukaryotic cell to which the bacterium of the invention is targeted can be any type of cell. A preferred cell is from a mucosal **tissue**. In one embodiment, the cell is a natural target of the bacterium. In another embodiment, the target cell is modified, e.g., genetically, to contain a surface receptor necessary for mediating the interaction between the bacterium and the target cell.

Brief Summary Text (27):
Thus, the method of the invention retains all the advantages and properties of introducing RNA into a eukaryotic cell and provides a more efficient manner to deliver the RNA to the target eukaryotic cell. The advantages of introducing RNA into a eukaryotic cell instead of a DNA molecule include (i) avoidance of risk of insertion of DNA into the genome of the target eukaryotic cell and thus strongly reduced risk of mutation of the target eukaryotic cell; (ii) absence of need for the nucleic acid introduced in the eukaryotic cell to traverse the nuclear membrane; and (iii) avoidance of the possibility of shedding of plasmid molecules from the bacteria. Delivery of RNA to eukaryotic cells by use of a bacterium, compared to delivery of "naked" RNA, e.g., where expression of the RNA is desired, provides at least the advantage that the RNA is protected and less likely to be degraded prior to entering the eukaryotic cell. Furthermore, the RNA can be specifically targeted to certain types of cells, since the bacterium can naturally target or be modified to target specific types of cells, e.g., antigen presenting

cells in the mucosal lymphoid **tissue**.

Brief Summary Text (28):
Furthermore, the invention provides methods and compositions for oral vaccines, in particular, for an oral mucosal HIV-1 vaccine. Historically, oral vaccines have proven to be an efficacious means to invoke mucosal immunity. The invention provides oral vaccines using Shigella bacteria, which possess specialized adaptations that allow this organism to invade and replicate in the cytoplasm of antigen presenting cells associated with the colonic lymphoid **tissue**, thus eliciting strong immune responses. Thus, the invention provides efficacious oral vaccines.

Detailed Description Text (19):
At least one advantage to Shigella RNA vaccine vectors is their tropism for lymphoid **tissue** in the colonic mucosal surface. In addition, the primary site of Shigella replication is believed to be within dendritic cells and macrophages, which are commonly found at the basal lateral surface of M cells in mucosal lymphoid **tissues** (reviewed by McGhee, J. R. et al. (1994) Reproduction, Fertility, & Development 6:369; Pascual, D. W. et al. (1994) Immunomethods 5:56). As such, Shigella vectors may provide a means to express antigens in these professional antigen presenting cells. Another advantage of Shigella vectors is that attenuated Shigella strains deliver nucleic acid reporter genes in vitro and in vivo (Sizemore, D. R. et al. (1995) Science 270:299; Courvalin, P. et al. (1995) Comptes Rendus de l Academie des Sciences Serie III-Sciences de la Vie-Life Sciences 318:1207; Powell, R. J. et al. (1996) In: Molecular approaches to the control of infectious diseases. F. Brown, E. Norrby, D. Burton and J. Mekalanos, eds. Cold Spring Harbor Laboratory Press, New York. 183; Anderson, R. J. et al. (1997) Abstracts for the 97th General Meeting of the American Society for Microbiology:E.). On the practical side, the tightly restricted host specificity of Shigella stands to prevent the spread of Shigella vectors into the food chain via intermediate hosts. Furthermore, attenuated strains that are highly attenuated in rodents, primates and volunteers have been developed (Anderson et al. (1997) supra; Li, A. et al. (1992) Vaccine 10:395; Li, A. et al. (1993) Vaccine 11:180; Karnell, A. et al. (1995) Vaccine 13:88; Sansonetti, P. J. and J. Arondel (1989) Vaccine 7:443; Fontaine, A. et al. (1990) Research in Microbiology 141:907; Sansonetti, P. J. et al. (1991) Vaccine 9:416; Noriega, F. R. et al. (1994) Infection & Immunity 62:5168; Noriega, F. R. et al. (1996) Infection & Immunity 64:3055; Noriega, F. R. et al. (1996) Infection & Immunity 64:23; Noriega, F. R. et al. (1996) Infection & Immunity 64:3055; Kotloff, K. L. et al. (1996) Infection & Immunity 64:4542). This latter knowledge will allow the development of well tolerated Shigella vectors for use in humans.

Detailed Description Text (21):
The attenuating mutations can be either constitutively expressed or under the control of **inducible promoters, such as the temperature** sensitive heat shock family of promoters (Neidhardt et al, supra), or the anaerobically **induced nirB promoter** (Harborne et al, Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as uapA (Gorfinkiel et al, J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer et al, J. Bact., 176:6159-6164 (1994)).

Detailed Description Text (53):
The above cytoplasm-targeting genes can be obtained by, e.g., PCR amplification from DNA **isolated** from an invasive bacterium carrying the desired cytoplasm-targeting gene. Primers for PCR can be designed from the nucleotide sequences available in the art, e.g., in the above-listed references and/or in GenBank, which is publically available on the internet (www.ncbi.nlm.nih.gov/). The PCR primers can be designed to amplify a cytoplasm-targeting gene, a cytoplasm-targeting operon, a cluster of cytoplasm-targeting genes, or a regulon of cytoplasm-targeting genes. The PCR strategy employed will depend on the genetic organization of the cytoplasm-targeting gene or genes in the target invasive bacteria. The PCR primers

are designed to contain a sequence that is homologous to DNA sequences at the beginning and end of the target DNA sequence. The cytoplasm-targeting genes can then be introduced into the target bacterial strain, e.g., by using Hfr transfer or plasmid mobilization (Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); Bothwell et al, supra; and Ausubel et al, supra), bacteriophage-mediated transduction (de Boer, supra; Miller, supra; and Ausubel et al, supra), chemical transformation (Bothwell et al, supra; Ausubel et al, supra), electroporation (Bothwel et al, supra; Ausubel et al, supra; and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and physical transformation techniques (Johnston et al, supra; and Bothwell, supra). The cytoplasm-targeting genes can be incorporated into lysogenic bacteriophage (de Boer et al, Cell, 56:641-649 (1989)), plasmids vectors (Curtiss et al, supra) or spliced into the chromosome (Hone et al, supra) of the target strain.

Detailed Description Text (55):
Bacteria can also be modified genetically to express an antibody, or variant thereof, or other factor capable of binding specifically to a receptor of an invasion factor, e.g., an integrin molecule. Antibody genes can be **isolated** according to methods known in the art. Furthermore, to reduce an immune reaction of the host towards the antibody, it may be desirable to use humanized antibodies. Such antibodies can be prepared as described in U.S. Pat. No. 5,585,089. Accordingly, the invention encompasses any bacterium modified, either genetically or otherwise, to have on its surface a factor, e.g., antibody, binding specifically to a receptor of an invasion factor, said bacterium being capable of delivering RNA to a eukaryotic cell.

Detailed Description Text (58):
Preferred target cells are eukaryotic cells. Even more preferred target cells are animal cells. "Animal cells" are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxanomic position lies within the kingdom animalia. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular **tissue** source of the cells is not critical to the present invention.

Detailed Description Text (62):
Although certain types of bacteria may have a certain tropism, i.e., preferred target cells, delivery of RNA to a certain type of cell can be achieved by choosing a bacterium which has a tropism for the desired cell type or which is modified such as to be able to invade the desired cell type. Thus, e.g., a bacterium could be genetically engineered to mimic mucosal **tissue** tropism and invasive properties, as discussed above, to thereby allow said bacteria to invade mucosal **tissue**, and deliver RNA to cells in those sites.

Detailed Description Text (88):
The term "eukaryotic RNA expression cassette" is used herein to define a nucleic acid which comprises elements or sequences which, when present in an RNA molecule, significantly increase its translation efficiency in a eukaryotic cell. For example, a CITE sequence is a preferred element of a eukaryotic RNA expression cassette. Another sequence which may be included in a eukaryotic RNA expression cassette is a polyadenylate sequence. The presence of a polyadenylate tail on an RNA may improve its **stability** both in the invasive bacterium and in the target eukaryotic cells, and may therefore be a desirable element.

Detailed Description Text (91):
In a preferred embodiment, the RNA contains upstream of the CITE sequence a nucleotide sequence which can prevent degradation of a CITE sequence by a 5'-3' exonuclease activity. While no such RNAse activity has been identified in enteric bacteria (reviewed in Kushner. "mRNA Decay" in Neidhhart et al. Eds. Escherichia coli and Salmonella. Cellular and Molecular Biology. Second ed. Vol. 1. Washington

D.C.: ASM Press, 1996:849), it may be desirable in certain situations to add a 5' nucleotide sequence which would significantly reduce degradation of the CITE sequence. A preferred nucleotide sequence that can be inserted upstream of the CITE sequence is at least a portion of a 5' untranlated region of OmpA, which forms a three stem-loop structure and has been shown to impart **stability** to RNA (Emory et al. (1992) Genes Dev. 6:135).

Detailed Description Text (93):
Thus, based at least on the possible presence of specific elements in, 5' or 3' untranslated regions of RNAs which influence **stability** and/or translation efficiency, constitutively, or in an inducible manner, the **stability** and/or translation efficiency of specific RNAs will vary depending on whether 5' and/or 3' untranslated regions are present. The effect of the presence of 5' and/or 3' untranslated regions or portions thereof in an RNA can be determined experimentally, e.g., by transfection experiments. For example, RNA **stability** may be determined by pulse labeling experiments. Accordingly, the invention provides methods for delivering RNA to eukaryotic cells, wherein the amount of protein synthesized in the eukaryotic cell from the RNA can be controlled by modifying the RNA such as to effect its translation efficiency and/or half-life.

Detailed Description Text (97):
The RNA may allow expression of either a foreign or an endogenous protein. As used herein, "foreign protein" or "heterologous protein" refers to a protein, which is foreign to the recipient eukaryotic cell or **tissue**, such as a vaccine antigen, immunoregulatory agent, or therapeutic agent. An "endogenous protein" refers to a protein which is naturally present in the recipient animal cell or **tissue**.

Detailed Description Text (107):
In the present invention, bacteria can also deliver RNA molecules encoding a therapeutic vaccine. As used herein, "therapeutic vaccine" refers to a vaccine comprising a therapeutic agent, which is a eukaryotic protein or peptide which is present or may be present in a subject, the shielding of which or elimination of which is desired. For example, the RNA molecules can encode a **tumor**-specific, transplant, or autoimmune antigen or part thereof.

Detailed Description Text (108):
Examples of **tumor** vaccine antigens include prostate specific antigen (Gattuso et al, Human Pathol., 26:123-126 (1995)), TAG-72 and carcinoembryonic antigen (CEA) (Guadagni et al, Int. J. Biol. Markers, 2:53-60 (1994)), MAGE-1 and tyrosinase (Coulie et al, J. Immunothera., 14:104-109 (1993)). Yet any other **tumor** antigen can also be used according to the method of the invention, to elicit an immune reaction against **tumor** cells expressing the antigen. It has in fact been shown in **mice** that immunization with non-malignant cells expressing a **tumor** antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant **tumor** cells displaying the same antigen (Koeppen et al, Anal. N.Y. Acad. Sci., 690:244-255 (1993)). Accordingly, **tumor** specific antigens can be delivered to a subject having a **tumor** or likely to develop a **tumor** according to the method of the invention to thereby induce an immune reaction against the **tumor** and either reduce or elimate the **tumor** or prevent a **tumor** from developing.

Detailed Description Text (110):
The method of the invention can also be used to destroy autoimmune cells. According to the method of the invention, an autoimmune antigen is administered to a subject prophylactically or therapeutically, to thereby result in the production of antibodies against the autoimmune antigen, thereby inducing destruction of autoimmune cells or preventing their development. Examples of autoimmune antigens include IAS .beta. chain (Topham et al, Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). In fact, it has been shown that vaccination of **mice** with an 18 amino acid peptide from IAS .beta. chain provides protection and treatment to **mice** with experimental autoimmune encephalomyelitis (Topham et al, supra).

Detailed Description Text (111):
Alternatively, in the present invention, bacteria can deliver RNA molecules encoding immunoregulatory molecules, e.g., to boost the immune response against said antigens. These immunoregulatory molecules include, but are not limited to, growth factors, such as M-CSF, GM-CSF, erythropoietin; and cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 or IFN-.gamma., clotting factors, **tissue** plasminogen activators, recombinant soluble receptors, e.g., IL-1 or TNF receptor. Furthermore, delivery of cytokines expression cassettes to tumorous **tissue** has been shown to stimulate potent systemic immunity and enhanced **tumor** antigen presentation without systemic cytokine toxicity (Golumbek et al, Canc. Res., 53:5841-5844 (1993); Golumbek et al, Immun. Res., 12:183-192 (1993); Pardoll, Curr. Opin. Oncol., 4:1124-1129 (1992); and Pardoll, Curr. Opin. Immunol., 4:619-623 (1992)).

Detailed Description Text (117):
In addition to the sequence complementary to a target sequence, the antisense RNA can comprise sequences which are capable of modulating the **stability** of the RNA. Accordingly, the same sequences set forth above for either enhancing or decreasing RNA **stability**, e.g., AU-rich sequences, can be linked to the antisense sequence per se. Alternatively, the portion of the antisense RNA which is complementary to the target sequence can be modified to eliminate specific sequences, e.g., sequences rendering the RNA unstable, so long as the antisense RNA is still capable of hybridizing to the target sequence. Modification of the antisense RNA can be performed according to methods known in the art, e.g., site directed mutagenesis.

Detailed Description Text (122):
As in the antisense approach, the ribozymes can be composed of modified RNAs (e.g., for improved **stability**). Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Detailed Description Text (138):
In yet another embodiment, the RNA is introduced into the bacterium for delivery to the eukaryotic cell. The RNA can be, e.g., synthesized in vitro using, e.g., an in vitro transcription system. Alternatively, the RNA can be **isolated** from a source, and can be, e.g., a mixture of different RNAs. In one embodiment, the RNA is RNA extracted from a pathogenic organism. In an even more preferred embodiment, a specific population of RNA from a pathogenic organism is selected, preferably a population of RNA which does not encode pathogenic proteins, and introduced in the bacterium. The RNA can be introduced into bacteria using the same methods for introducing DNA into bacteria. For example, RNA can be introduced by electroporation or chemical transformation.

Detailed Description Text (155):
The invasive bacteria containing the RNA expression cassette can be used to infect animal cells that are cultured in vitro, such as cells obtained from a subject. These in vitro-infected cells can then be introduced into animals, e.g., the subject from which the cells were obtained initially, intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host **tissue**. When delivering RNA to individual cells, the dosage of viable organisms to administered will be at a multiplicity of infection ranging from about 0.1 to 10.sup.6, preferably about 10.sup.2 to 10.sup.4 bacteria per cell.

Detailed Description Text (156):
In yet another embodiment of the present invention, bacteria can also deliver RNA molecules encoding proteins to cells, e.g., animal cells, from which the proteins can later be harvested or purified. For example, a protein can be produced in a **tissue** culture cell.

Detailed Description Text (157):

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the **Mouse** Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Detailed Description Text (167):
Expression of .beta.-galactosidase-encoding RNA in the transformed bacteria was confirmed by RNA hybridization using the RNeasy.TM. total RNA **isolation** system according to the manufacturer's instructions (Qiagen).

Detailed Description Text (170):
HeLa cells (ATCC No. CCL-2) were grown on plastic **tissue** culture plates at 37.degree. C. in 5% (v/v) CO.sub.2 in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2.0 mM L-glutamine, 1.0 mM L-pyruvate, 50 U/ml penicillin and 50 .mu.g/ml streptomycin (hereinafter "RPMI/FBS"). 24 to 48 hours prior to RNA delivery, the HeLa cells were trypsinized with 0.25% (w/v) trypsin containing 1.0 mM EDTA, and split by limiting dilution such that they were 70-90% confluent at the time of the experiment.

Detailed Description Text (190):
To evaluate the delivery of the GFP RNA expression cassette in vivo, a 10.sup.6 cfu dose of strain H992 or V-C1 was injected intraperitoneally into 6-8 week old female BALB/c **mice**. 24 hr after treatment cells expressing GFP were clearly evident in mononuclear cells harvested from the peritoneum of **mice** treated with H992. On the other hand, no discernable GFP expression was observed in **mice** treated with control strain V-C1.

Detailed Description Text (200):
This example describes the characterization of the immunogenicity of Shigella strain CVD 1203 bearing both plasmids, pGP1-2 and pT7-CITE::120 (strain referred to as H1015) in BALB/c **mice** in which the RNA vaccine vector was inoculated intranasally. Control **mice** received CVD 1203 containing pT7-CITE::120 only (referred to as H1011). Groups of 5 BALB/c **mice** were vaccinated intranasally with a single 10.sup.6 dose of strains H1011 or H1015 as described ((van de Verg et al. (1995) Infection & Immunity 63(5): 1947-54)).

Detailed Description Text (201):
To measure gp120-specific immune responses, the **mice** were sacrificed 28 days after vaccination, and splenocytes (SCs) were prepared as described (Foutset al. (1995) Vaccine 13(17): 1697-705). The presence and the amount of V3-specific CD8+ T cells was then determined using an IFN-.gamma.-specific ELISPOT assay (Chada et al.

(1993) Journal of Virology 67(6): 3409-17). In this assay, 10ME fibroblasts which were pulsed with peptide P18, which is located in the V3 region (Takeshita et al. (1995) Journal of Immunology 154(4): 1973-86), were used as stimulators. The results of this assay showed that gp120 RNA vaccine strain H1015 elicited a net 37.5 V3-specific IFN-.gamma.-secreting T cells per 10.sup.6 splenocyte above the baseline V3-specific IFN-.gamma.-secreting T cell response established by vaccination with the control strain.

Detailed Description Text (202):
Depletion of CD8+ T cells but not of CD4+ T cells from the immune splenocytes resulted in a loss of the V3-specific responsiveness in the splenocytes from the **mice** vaccinated with H1015, indicating that the response was largely mediated by CD8+ T cells. This result is encouraging in light of the extensive data implicating CD8+ T cells in the control of, and protection against, HIV-1 ((Walker et al. (1988) Science 240(4848): 64-6; Walker and Levy (1989) Immunology 66(4): 628-30; Walker et al. (1989) Cell Immunol 119(2): 470-5; Shen et al. (1991) Science 252 (5004): 440-3; Walker, C. M. (1993) Seminars in Immunology 5(3):195-201; Walker et al. (1991) J Virol 65(11): 5921-7; Abimiku et al. (1995) AIDS Research & Human Retroviruses 11(3): 383-93; Cocchi et al. (1995) Science 270(5243): 1811-5; Lohman et al. (1995) Journal of Immunology 155(12): 5855-60; Miller et al. (1997) Journal of Virology 71(3): 1911-21; Garzino-Demo et al. (1998) AIDS Res Hum Retroviruses 14 Suppl 2: S177-84; Price et al. (1998) Curr Biol 8(6): 355-8)).

Detailed Description Text (209):
Following transformation, the plasmids are characterized by restriction endonuclease digestion and PCR analysis as above to verify that the correct configurations are present in each strain. In addition, expression of Env-encoding RNA by each construct is confirmed by RNA hybridization using the RNeasy.sup.R total RNA **isolation** system according to the manufacturer's instructions (Qiagen). Strains that display the appropriate genotype and RNA profile can be subsequently used to evaluate the efficacy of such a plasmid-based RNA delivery system in vitro and in vivo.

Detailed Description Text (217):
Delivery of a Reporter Gene in vivo to Animal **Tissue**

Detailed Description Text (218):
This Example describes in vivo assays in **mice** to demonstrate the immunogenicity of a Shigella RNA vaccine delivery system by monitoring humoral and cellular immune responses in both the mucosal and systemic compartments. Pretreatment of **mice** with streptomycin alters the intestinal flora and creates a favorable environment for Shigella invasion of the **mouse** gastrointestinal tract (Cooper (1959) Australian J. Exp. Biol. Med. Sci. 37:193). Thus, this model offers inexpensive means to evaluate RNA vaccine delivery to these **tissues**. The effectiveness of this delivery system will then be fully investigated in Rhesus monkeys and volunteers.

Detailed Description Text (219):
BALB/c **mice**, 6-8 weeks old, will be housed in sterilized microisolator cages and maintained on sterile food and water containing streptomycin (1 mg/ml) (Cooper, supra). Strains bearing pGB-T7 will be streptomycin resistant but the chromosomal constructs will be sensitive to this antibiotic. Therefore, the chromosomal constructs must first be made streptomycin-resistant prior to the murine studies. This will be accomplished by **isolating** spontaneous streptomycin-resistant derivatives of these constructs, which are selected on solid agar containing 1 mg/ml streptomycin. To vaccinate the animals, each **mouse** will be given 0.2 ml of 50% saturated bicarbonate solution by orogastric intubation as described in Hone et al. (1987) J. Infect. Diseases 156:167.

Detailed Description Text (220):
Two separate experiments will be conducted: (1) To evaluate the induction of

humoral responses, groups of 5 BALB/c **mice** will be immunized by orogastric intubation with a single 10.sup.9 cfu dose of the Shigella constructs. (2) To evaluate T cell-mediated responses, groups of 35 BALB/c **mice** will be immunized by orogastric intubation with a single 10.sup.9 cfu dose of the Shigella constructs. These two experimental groups will be boosted 30, 58, and 86 days after the primary dose. The first group will be immunized with Shigella strains containing either of both of the plasmids pGB-T7 and pCITE4:env.sub.MN, prepared as described above. The second group will be immunized with Shigella strains having the plasmids pGB-T7 and pCITE4:env.sub.MN, or with Shigella strains having either or both of plasmids pnirB-PolT7 and pT7-CITE-envMN, prepared as described above. Positive control **mice** will be immunized intranasally with 10 .mu.g HIV-1.sub.MN Env mixed with 5 .mu.g cholera toxin, which induces strong Env-specific immunity.

Detailed Description Text (222):
Blood will be collected from the tail veins of vaccinated **mice** 14, 28, 42, 56, 70, 84, 98, and 112 days after the primary immunization. The level of Env-specific IgG and IgA will be measured in sera separated from these blood samples by ELISA using purified fully glycosylated HIV-1.sub.MN Env as described, e.g., in Abacioglu et al. (1994) AIDS Res. & Human Retroviruses 10:371 or in Moore et al. (1994) J. Virol. 68:6836 In each ELISA, Env-specific mAbs will be used as a positive control (Abacioglu et al., supra; Moore et al., supra; and Moore (1990) AIDS 4:297). In addition, these sera will be used to follow the development of HIV-1 neutralizing antibodies by the quantitative, linear HIV-1 infectivity assay as described, e.g., in Nara et al. (1990) J. Virol. 64:3779, Layne et al. (1991) Virol. 189:695, Layne et al. (1991) J. Virol. 65:3291, and Wu et al. (1995) J. Virol. 69:6054, incorporating HIV-1.sub.MN (Gurgo et al. (1988) Virol. 164:531) to measure homologous neutralization and HIV-1.sub.IIIB, HIV-1.sub.RF as well as primary HIV-1 **isolates** (from the AIDS Repository, NIAID) to measure clade-specific and cross-clade neutralization (Nara et al, supra, Layne et al., supra, Wu et al, supra).

Detailed Description Text (223):
(ii) **Tissue** Harvesting for T Cell Assays and Env-specific Antibody Secreting Cells (ASC) in the Lamina Propria

Detailed Description Text (224):
Lamina propria antigen-specific antibody secreting cells are a useful and convenient measure of mucosal immunity, since these cells are T cell-dependent and the level of the response correlates with the development of humoral responses at the mucosal surface after immunization. Groups of 5 **mice** will be sacrificed 7, 14, 28, 42, 56, and 70 days after primary immunization and mononuclear cells (MNCs) from the **spleen**, small intestine and mesenteric lymph nodes will be prepared as described (Wu S. et al. (1995) Infection & Immunity 63:4933-8; Fouts TR et al. (1995) Vaccine 13:1697-705; Okahashi N. et al. (1996) Infection & Immunity 64:1516-25; Xu-Amano J. et al. (1993) J. Exp. Med. 178:1309-20).

Detailed Description Text (229):
In addition, MNCs will be cultured in complete medium (CM) only, CM containing fully glycosylated HIV-1.sub.MN Env at 0.1-10 or CM containing Shigella outer membrane antigen (10 .mu.g/ml) as shown above. The culture plates will be incubated then for 24 hr at 37.degree. C. in 5% CO.sub.2. After stimulation, CD4.sup.+ and CD8.sup.+ T cells will be **isolated** by flow cytometry and no fewer than 5.times.10.sup.5 of the purified cells will be placed directly into Trizol.sup.R reagent and cDNA will be synthesized; the resultant samples then will be used in quantitative-competitive (QC) PCR reactions to evaluate the relative levels of chemokine and cytokine cDNA sequences. MIP-1, MIP-1, RANTES, TNF, IL-2, IL-4, IL-5, IL-6, and IFN-.gamma. sequence specific RT-PCR primers are designed based on the known sequences of these molecules, e.g., GenBank. Each QC PCR reaction will be conducted in parallel with the same reactions containing of control plasmid DNA at a range of defined concentrations, which encode truncated MIP-1, MIP-1 and RANTES cDNA sequences and will serve as competitive sequences (R&D Systems and National

Biosciences Inc). The PCR fragments will be separated by agarose gel electrophoresis, strained with ethidium bromide and scanned using a BioRad UV densitometer. The results will be expressed as arbitrary mRNA units.

Detailed Description Text (232):
To characterize HIV-specific chemokine-secreting CD8.sup.+ T cell responses, a further group of **mice** will be immunized as above. On days 7, 14, 28, 42, 56, and 70 after immunization, groups of 5 **mice** will be sacrificed and MNCs from the **spleens**, the intestinal epithelial layer and lamina propria, and mesenteric lymph nodes will be prepared as described above. The MNCs will be divided into total, CD4.sup.+ T cell-depleted and CD8.sup.+ T cell-depleted cells, and will be cultured in (i) CM only, (ii) CM containing PHA (Flynn J L et al. (1990) Molecular Microbiology 4:2111-8), (iii) CM containing BC-lacZ fibroblasts expressing galactosidase (Aggarwal A. et al. (1990) J. Exp. Med. 172:1083-90) or (iv) CM containing BC-env fibroblasts expressing gp160 (Abimiku, A G et al. (1995) AIDS Research & Human Retroviruses 11:383-93). The stimulator cells will be titrated in the range of 1000 to 500000 cells per well. The cells then will incubated for 72 hr at 37.degree. C. in 5% CO.sub.2 and supernatants will be collected every 24 hr and stored at -80.degree. C. These supernatants will be used to quantitate the levels of MIP-1, MIP-1, TNF-.alpha., IL-2, IL-4, IL-5, IL-6 and IFN-.gamma. by ELISA (see above). In addition, after stimulation CD8.sup.+ T cells will be **isolated** from total MNC cultures by flow cytometry to obtain a minimum of 5.times.10.sup.5 cells, which will be placed directly into Trizol.sup.R reagent and cDNA will be synthesized; QC PCR reactions will be used to evaluate the relative levels of chemokine and cytokine cDNA.

Detailed Description Text (234):
After 5 days of such stimulation HIV-specific CTL activity will be measured at both mucosal and systemic effector sites with methodology appropriate to the site (Chada S. et al. (1993) J. Virol. 67:3409-17; Fujihashi K. et al. (1990) J. Immunol. 145:2010-9): the **spleen** will serve as the systemic site and intestinal intraepithelial and lamina propria lymphocytes will serve as the mucosal sites. CTL activity will be measured using targets .sup.51 Cr-labeled (i) BC-lacZ fibroblast or (ii) BC-env fibroblast (Chada S. et al., supra; Fujihashi K. et al., supra). When killing is observed, the effector cells will be phenotyped using standard methods applied by our group previously (Aggarwal A. et al., supra). For quantitative comparisons, CTL responses will be expressed in lytic units/10.sup.6 cells.

CLAIMS:

8. The live-invasive bacterium of claim 1, wherein said bacterium is selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

9. The live-invasive bacterium of claim 1, wherein said bacterium is selected from the group consisting of Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., Leishmania spp. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

29. The method of claim 19, wherein said invasive bacteria are selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

30. The method of claim 19, wherein said invasive bacteria are selected from the

group consisting of Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., Leishmania spp. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

42. The method of claim 37, wherein said bacterium is selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia app and enteroinvasive Escherichia coli.

43. The method of claim 37, wherein said bacterium is selected from the group consisting of Yersinia spp., Eacherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter app., Salmonella app., Vibrio spp., Bacillus spp., Leishmania app. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

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L29: Entry 15 of 16 File: USPT Nov 21, 2000

DOCUMENT-IDENTIFIER: US 6150170 A
TITLE: Method for introducing and expressing genes in animal cells, and live invasive bacterial vectors for use in the same

Brief Summary Text (2):
The present invention relates to a method for introducing endogenous or foreign genes into animal cells using live invasive bacteria as vectors. The method allows for the delivery of eukaryotic expression cassettes encoding the endogenous or foreign genes into animal cells or animal **tissue**, and is useful for expressing, e.g., vaccine antigens, therapeutic agents, immunoregulatory agents, antisense RNAs, and catalytic RNAs, in animal cells or animal **tissue**.

Brief Summary Text (5):
The advent of recombinant DNA technology has greatly accelerated the development of vaccines to control epidemic, endemic, and pandemic infectious diseases (Woodrow et al, New Generation Vaccines: The Molecular Approach, Eds., Marcel Dekker, Inc., New York, N.Y. (1989); Cryz, Vaccines and Immunotherapy, Ed., Pergamon Press, New York, N.Y. (1991); and Levine et al, Ped. Ann., 22:719-725 (1993)). In particular, this technology has enabled the growth of a new class of vaccines called bacterial vector vaccines (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, N.Y., pages 161-188 and 269-288 (1989); and Mims et al, In: Medical Microbiology, Eds., Mosby-Year Book Europe Ltd., London (1993)). These vaccines can enter the host, either orally, intranasally or parenterally. Once gaining access to the host, the bacterial vector vaccines express an engineered prokaryotic expression cassette contained therein that encodes a foreign antigen(s). Foreign antigens can be any protein (or part of a protein) or

combination thereof from a bacterial, viral, or parasitic pathogen that has vaccine properties (New Generation Vaccines: The Molecular Approach, supra; Vaccines and Immunotherapy, supra; Hilleman, Dev. Biol. Stand., 82:3-20 (1994);.degree. Formal et al, Infect. Immun. 34:746-751 (1981); Gonzalez et al, J. Infect. Dis., 169:927-931 (1994); Stevenson et al, FEMS Lett., 28:317-320 (1985); Aggarwal et al, J. Exp. Med., 172:1083-1090 (1990); Hone et al, Microbial. Path., 5:407-418 (1988); Flynn et al, Mol. Microbiol., 4:2111-2118 (1990); Walker et al, Infect. Immun., 60:4260-4268 (1992); Cardenas et al, Vacc., 11:126-135 (1993); Curtiss et al, Dev. Biol. Stand., 82:23-33 (1994); Simonet et al, Infect. Immun., 62:863-867 (1994); Charbit et al, Vacc., 11:1221-1228 (1993); Turner et al, Infect. Immun., 61:5374-5380 (1993); Schodel et al, Infect. Immun., 62:1669-1676 (1994); Schodel et al, J. Immunol., 145:4317-4321 (1990); Stabel et al, Infect. Immun., 59:2941-2947 (1991); Brown, J. Infect. Dis., 155:86-92 (1987); Doggett et al, Infect. Immun., 61:1859-1866 (1993); Brett et al, Immunol., 80:306-312 (1993); Yang et al, J. Immunol., 145:2281-2285 (1990); Gao et al, Infect. Immun., 60:3780-3789 (1992); and Chatfield et al, Bio/Technology, 10:888-892 (1992)). Delivery of the foreign antigen to the host **tissue** using bacterial vector vaccines results in host immune responses against the foreign antigen, which provide protection against the pathogen from which the foreign antigen originates (Mims, The Pathogenesis of Infectious Disease, Academic Press, London (1987); and New Generation Vaccines: The Molecular Approach, supra).

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Successful delivery of DNA to animal **tissue** has been achieved by cationic liposomes (Watanabe et al, Mol. Reprod. Dev., 38:268-274 (1994)), direct injection of naked DNA into animal muscle **tissue** (Robinson et al, Vacc., 11:957-960 (1993); Hoffman et al, Vacc., 12:1529-1533; (1994); Xiang et al, Virol., 199:132-140 (1994); Webster et al, Vacc., 12:1495-1498 (1994); Davis et al, Vacc., 12:1503-1509 (1994); and Davis et al, Hum. Molec. Gen., 2:1847-1851 (1993)), and embryos (Naito et al, Mol. Reprod. Dev., 39:153-161 (1994); and Burdon et al, Mol. Reprod. Dev., 33:436-442 (1992)), or intradermal injection of DNA using "gene gun" technology (Johnston et al, supra). A limitation of these techniques is that they only efficiently deliver DNA to parenteral sites. At present, effective delivery of eukaryotic expression cassettes to mucosal **tissue** has been met with limited success. This is presumably due to poor access to these sites, toxicity of the delivery vehicles or instability of the delivery vehicles when delivered orally.

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The delivery of endogenous and foreign genes to animal **tissue** for gene therapy has shown significant promise in experimental animals and volunteers (Nabel, Circulation, 91:541-548 (1995); Coovert et al, Curr. Opin. Neuro., 7:463-470 (1994); Foa, Bill. Clin. Haemat., 7:421-434 (1994); Bowers et al, J. Am. Diet. Assoc., 95:53-59 (1995); Perales et al, Eur. J. Biochem., 226:255-266 (1994); Danko et al, Vacc., 12:1499-1502 (1994); Conry et al, Canc. Res., 54:1164-1168 (1994); and Smith, J. Hemat., 1:155-166 (1992)). Recently, naked DNA vaccines carrying eukaryotic expression cassettes have been used to successfully immunize against influenza both in chickens (Robinson et al, supra) and ferrets (Webster et al, Vacc., 12:1495-1498 (1994)); against Plasmodium yoelii in **mice** (Hoffman et al, supra); against rabies in **mice** (Xiang et al, supra); against human carcinoembryonic antigen in **mice** (Conry et al, supra) and against hepatitis B in **mice** (Davis et al, supra). These observations open the additional possibility that delivery of endogenous and foreign genes to animal **tissue** could be used for prophylactic and therapeutic applications.

Brief Summary Text (24):
Therefore, there is a need to deliver eukaryotic expression cassettes, encoding endogenous or foreign genes that are vaccines or therapeutic agents to animal cells or **tissue**. In particular, a method that delivers eukaryotic expression cassettes to mucosal surfaces is highly desirable. Bacterial vector vaccines have been used in the past to deliver foreign antigens encoded on prokaryotic expression cassettes to

animal **tissue** at mucosal sites.

Brief Summary Text (25):
The present invention describes a novel and unexpected finding that invasive bacteria are capable of delivering eukaryotic expression cassettes to animal cells and **tissue**. An important aspect of using live invasive bacteria to deliver eukaryotic expression cassettes is that they are capable of delivering DNA to mucosal sites.

Brief Summary Text (26):
Heretofore, there has been no documented demonstration of live bacteria invading animal cells and introducing a eukaryotic expression cassette(s), which then is expressed by the infected cells and progeny thereof. That is, the present invention provides the first documentation of genetic exchange between live invasive bacteria and animals cells. Heretofore, foreign antigen delivery by live bacterial vector vaccines merely involved delivery of prokaryotic expression cassettes to and expression of the foreign antigen by the bacterial vaccine vector, in animal cells or **tissues**. In contrast, the present invention involves the delivery of eukaryotic expression cassettes by live bacterial strains to animal cells in vitro or to cells in animal **tissue**, and expression of the eukaryotic expression cassettes by the animal cell or cells in animal **tissue**.

Brief Summary Text (28):
An object of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes to animal cells or animal **tissue**.

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Another object of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes encoding a vaccine antigen(s) to animal cells or animal **tissue**.

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Brief Summary Text (31):
Yet another objective of the present invention is to use live invasive bacteria to deliver one or more eukaryotic expression cassettes encoding biologically active RNA species to animal cells or animal **tissue**.

Detailed Description Text (3):
Animal cells are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxanomic position lies within the kingdom animalia. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular **tissue** source of the cells is not critical to the present invention.

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As used herein, "invasive bacteria" are bacteria that are capable of delivering eukaryotic expression cassettes to animal cells or animal **tissue**. "Invasive bacteria" include bacteria that are naturally capable of entering the cytoplasm or nucleus of animal cells, as well as bacteria that are genetically engineered to enter the cytoplasm or nucleus of animal cells or cells in animal **tissue**.

Detailed Description Text (30):
The attenuating mutations can be either constitutively expressed or under the control of **inducible promoters, such as the temperature** sensitive heat shock family of promoters (Neidhardt et al, supra), or the anaerobically **induced nirB promoter** (Harborne et al, Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as

uapA (Gorfinkiel et al, J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer et al, J. Bact., 176:6159-6164 (1994)).

Detailed Description Text (68):
Alternatively, any bacteria could be genetically engineered to mimic mucosal **tissue** tropism and invasive properties, as discussed above, that thereby allow said bacteria to invade mucosal **tissue**, and deliver genes at those sites.

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It is also possible to change the **tissue** specificity of the invasive bacteria by expression of a gene product singularly or in combination, e.g., the Plasmodium vivax reticulocyte binding proteins-1 and -2 bind specifically to erythrocytes in humans and primates (Galinski et al, Cell, 69:1213-1226 (1992)); Yersinia Invasin recognizes .beta.1 integrin receptors (Isberg et al, Trends Microbiol., 2:10-14 (1994)); asialoorosomucoid is a ligand for the asilogycoprotein receptor on hepatocytes (Wu et al, J. Biol. Chem., 263:14621-14624 (1988)); presence of insulin-poly-L-lysine has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz et al, Expt. Cell Res., 199:323-329 (1992)); p60 of Listeria monocytogenes allows for tropism for hepatocytes (Hess et al, Infect. Immun., 63:2047-2053 (1995)) and Trypanosoma cruzi expresses a 60 kDa surface protein which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria et al, Cell, 67:411-421 (1991)).

Detailed Description Text (72):
These cassettes usually are in the form of plasmids, and contain various promoters well-known to be useful for driving expression of genes in animal cells, such as the viral derived SV40, CMV and, RSV promoters or eukaryotic derived .beta.-casein, uteroglobin, .beta.-actin or tyrosinase promoters. The particular promoter is not critical to the present, except in the case where the object is to obtain expression in only selective cell types. In this case, the promoter is selected to be one which is only active in the selected cell type. Examples of **tissue** specific promoters include, but are not limited to, .alpha. S1- and .beta.-casein promoters which are specific for mammary **tissue** (Platenburg et al, Trans. Res., 3:99-108 (1994) ; and Maga et al, Trans. Res., 3:36-42 (1994)); the phosphoenolpyruvate carboxykinase promoter which is active in **liver**, kidney, adipose, jejunum and mammary **tissue** (McGrane et al, J. Reprod. Fert., 41:17-23 (1990)); the tyrosinase promoter which is active in **lung and spleen** cells, but not testes, **brain**, heart, **liver** or kidney (Vile et al, Canc. Res., 54:6228-6234 (1994)); the involucerin promoter which is only active in differentiating keratinocytes of the squamous epithelia (Carroll et al, J. Cell Sci., 103:925-930 (1992)); and the uteroglobin promoter which is active in **lung** and endometrium (Helftenbein et al, Annal. N.Y. Acad. Sci., 622:69-79 (1991)).

Detailed Description Text (73):
Alternatively, cell specific enhancer sequences can be used to control expression, for example human neurotropic papovirus JCV enhancer regulates viral transcription in glial cells alone (Remenick et al, J. Virol., 65:5641-5646 (1991)). Yet another way to control **tissue** specific expression is to use a hormone responsive element (HRE) to specify which cell lineages a promoter will be active in, for example, the MMTV promoter requires the binding of a hormone receptor, such as progesterone receptor, to an upstream HRE before it is activated (Beato, FASEB J., 5:2044-2051 (1991); and Truss et al, J. Steroid Biochem. Mol. Biol., 41:241-248 (1992)).

Detailed Description Text (75):
In the present invention, the live invasive bacteria can deliver eukaryotic expression cassettes encoding a gene into an animal cell or animal **tissue**. The gene may be either a foreign gene or a endogenous gene. As used herein, "foreign gene" means a gene encoding a protein or fragment thereof or anti-sense RNA or catalytic RNA, which is foreign to the recipient animal cell or **tissue**, such as a vaccine

antigen, immunoregulatory agent, or therapeutic agent. An "endogenous gene" means a gene encoding a protein or part thereof or anti-sense RNA or catalytic RNA which is naturally present in the recipient animal cell or **tissue**.

Detailed Description Text (85):
In the present invention, the live invasive bacteria can also deliver eukaryotic expression cassettes encoding a therapeutic agent to animal cells or animal **tissue**. For example, the eukaryotic expression cassettes can encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof. Alternatively, the eukaryotic expression cassettes can encode synthetic genes, which encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof.

Detailed Description Text (86):
Examples of **tumor** specific antigens include prostate specific antigen (Gattuso et al, Human Pathol., 26:123-126 (1995)), TAG-72 and CEA (Guadagni et al, Int. J. Biol. Markers, 9:53-60 (1994)), MAGE-1 and yrosinase (Coulie et al, J. Immunothera., 14:104-109 (1993)). Recently it has been shown in **mice** that immunization with non-malignant cells expressing a **tumor** antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant **tumor** cells displaying the same antigen (Koeppen et al, Anal. N.Y. Acad. Sci., 690:244-255 (1993)).

Detailed Description Text (88):
Examples of autoimmune antigens include IAS .beta. chain (Topham et al, Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). Vaccination of **mice** with an 18 amino acid peptide from IAS .beta. chain has been demonstrated to provide protection and treatment to **mice** with experimental autoimmune encephalomyelitis (Topham et al, supra).

Detailed Description Text (89):
Alternatively, in the present invention, live invasive bacteria can deliver eukaryotic expression cassettes encoding immunoregulatory molecules. These immunoregulatory molecules include, but are not limited to, growth factors, such as N-CSF, GM-CSF; and cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 or IFN-.gamma.. Recently, delivery of cytokines expression cassettes to **tumor tissue** has been shown to stimulate potent systemic immunity and enhanced **tumor** antigen presentation without producing a systemic cytokine toxicity (Golumbek et al, Canc. Res., 53:5841-5844 (1993); Golumbek et al, Immun. Res., 12:183-192 (1993); Pardoll, Curr. Opin. Oncol., 4:1124-1129 (1992); and Pardoll, Curr. Opin. Immunol., 4:619-623 (1992)).

Detailed Description Text (91):
In the present invention, live invasive bacteria can also deliver eukaryotic expression cassettes encoding proteins to animal **tissue** from which they can later be harvested or purified. An example is the delivery of a eukaryotic expression cassette under the control of a mammary specific viral promoter, such as derived from **mouse** mammary **tumor** virus (ATCC No. VR731), encoding .alpha..sub.1 -antitrypsin to mammary **tissue** of a goat or sheep.

Detailed Description Text (92):
Alternatively an invasive bacteria carrying a eukaryotic expression cassette can be introduced to a **tissue** site such that it would not spread from such a site. This could be accomplished by any of several methods including delivery of a very limited dose, delivery of a severely attenuated auxotrophic strain, such as an asd mutant (Curtiss et al, supra) that will be rapidly inactivated or die, or delivery of a bacterial strain that contains attenuating lesions, such as a suicide systems (Rennell et al, supra; and Reader et al, supra) under the control of a strong promoter, such as the anerobic nirB promoter (Harborne et al, supra) which will be switched on within the recipient host **tissue**. Additionally, through use of different species and/or serotypes multiple doses of invasive bacteria, the

eukaryotic expression cassette of interest can be given to an animal so as to manipulate expression levels or product type. This approach obviates the need for specially raised transgenic animals containing **tissue** specific promoters and having tight control of expression, as is currently the case (Janne et al, Int. J. Biochem., 26:859-870 (1994); Mullins et al, Hyperten., 22:630-633 (1993); and Persuy et al, Eur. J. Bichem., 205:887-893 (1992)).

Detailed Description Text (93):
As a further alternative, single or multiple eukaryotic expression cassettes encoding **tumor**-specific, transplant, and/or autoimmune antigens, can be delivered in any single or multiple combination with eukaryotic expression cassettes encoding immunoregulatory molecules or other proteins.

Detailed Description Text (95):
The invasive bacteria containing the eukaryotic expression cassette can be used to infect animal cells that are cultured in vitro. The animal cells can be further cultured in vitro, and the cells carrying the desired genetic trait can be enriched by selection for or against any selectable marker introduced to the recipient cell at the time of bactofection. Such markers may include antibiotic resistance genes, e.g., hygromycin, or neomycin, selectable cell surface markers, or any other phenotypic or genotypic element introduced or altered by bactofection. These in vitro-infected cells or the in vitro-enriched cells can then be introduced into animals intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host **tissue**.

Detailed Description Text (110):
HeLa cells (ATCC No. CCL-2) were grown on plastic **tissue** culture plates at 37.degree. C. in 5% (v/v) CO.sub.2 in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2.0 mM L-glutamine, 1.0 mM L-pyruvate, 50 U/ml penicillin and 50 .mu.g/ml streptomycin (hereinafter "RPMI/FBS"). 24 to 48 hours prior to bactofection, the HeLa cells were trypsinized with 0.25% (w/v) trypsin containing 1.0 mM EDTA, and split by limiting dilution such that they were 40-60% confluent at the time of the experiment.

Detailed Description Text (128):
Thus, it is evident that the method of the present invention is not restricted to one animal cell type, but is applicable to animal cells derived from various **tissues**.

Detailed Description Text (152):
Delivery of a Reporter Gene In Vivo to Animal **Tissue**

Detailed Description Text (153):
In order to demonstrate that bactofection can occur in vivo, restrained **mice** (Balb/c) were intranasally inoculated with 5.times.10.sup.6 viable S. flexneri .DELTA.aro.DELTA.virG containing either p.beta.-gal+SV or p.beta.-gal-SV in a volume of 10 .mu.l of PBS. 48 hours after inoculation, the **mice** were sacrificed, **lung tissue** collected and frozen to -70.degree. C. Cryosections (5.0 .mu.M) were prepared, fixed, and then stained overnight for .beta.-gal activity as described above (Hawley-Nelson et al, supra). Following staining, the sections were rinsed twice with PBS, then sealed under coverslips.

Detailed Description Text (154):
Blue-staining .beta.-gal-positive cells were visible per **lung** section infected with p.beta.-gal+SV, but not those infected with p.beta.-gal-SV.

Detailed Description Text (164):
In order to show another example of in vivo use of bactofection, 5.times.10.sup.7 S. flexneri .DELTA.aro .DELTA.virG containing the pCEP4::gp160 plasmid construct were administered intranasally to restrained Balb/c **mice**. 14 days following

bactofection, the **mice** were sacrificed and **spleens** collected.

Detailed Description Text (166):
Splenocytes **isolated from mice** bactofected with plasmid pCEP4::gp160, containing the gene for HIV-1 gp160, showed a seven-fold stimulation, while splenocytes from control (pCEP4) bactofected **mice** showed no response.

CLAIMS:

5. The method of claim 1, wherein said invasive bacteria are selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

9. The method of claim 1, wherein said invasive bacteria are selected from the group consisting of Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas Spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., Leishmania spp. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

16. Document ID: US 5877159 A

L29: Entry 16 of 16 File: USPT Mar 2, 1999

DOCUMENT-IDENTIFIER: US 5877159 A
TITLE: Method for introducing and expressing genes in animal cells and live invasive bacterial vectors for use in the same

Brief Summary Text (2):
The present invention relates to a method for introducing endogenous or foreign genes into animal cells using live invasive bacteria as vectors. The method allows for the delivery of eukaryotic expression cassettes encoding the endogenous or foreign genes into animal cells or animal **tissue**, and is useful for expressing, e.g., vaccine antigens, therapeutic agents, immunoregulatory agents, antisense RNAs, and catalytic RNAs, in animal cells or animal **tissue**.

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The advent of recombinant DNA technology has greatly accelerated the development of vaccines to control epidemic, endemic, and pandemic infectious diseases (Woodrow et al, New Generation Vaccines: The Molecular Approach, Eds., Marcel Dekker, Inc., New York, N.Y. (1989); Cryz, Vaccines and Immunotherapy, Ed., Pergamon Press, New York, N.Y. (1991); and Levine et al, Ped. Ann., 22:719-725 (1993)). In particular, this technology has enabled the growth of a new class of vaccines called bacterial vector vaccines (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, N.Y., pages 161-188 and 269-288 (1989); and Mims et al, In: Medical Microbiology, Eds., Mosby-Year Book Europe Ltd., London (1993)). These vaccines can enter the host, either orally, intranasally or parenterally. Once gaining access to the host, the bacterial vector vaccines express an engineered prokaryotic expression cassette contained therein that encodes a foreign

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It is also possible to change the **tissue** specificity of the invasive bacteria by expression of a gene product singularly or in combination, e.g., the Plasmodium vivax reticulocyte binding proteins-1 and -2 bind specifically to erythrocytes in humans and primates (Galinski et al, Cell, 69:1213-1226 (1992)); Yersinia Invasin recognizes .beta.1 integrin receptors (Isberg et al, Trends Microbiol., 2:10-14 (1994)); asialoorosomucoid is a ligand for the asilogycoprotein receptor on hepatocytes (Wu et al, J. Biol. Chem., 263:14621-14624 (1988)); presence of insulin-poly-L-lysine has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz et al, Expt. Cell Res., 199:323-329 (1992)); p60 of Listeria monocytogenes allows for tropism for hepatocytes (Hess et al, Infect. Immun., 63:2047-2053 (1995)) and Trypanosoma cruzi expresses a 60 kDa surface protein which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria et al, Cell, 67:411-421 (1991)).

Detailed Description Text (71):
These cassettes usually are in the form of plasmids, and contain various promoters well-known to be useful for driving expression of genes in animal cells, such as the viral derived SV40, CMV and, RSV promoters or eukaryotic derived .beta.-casein, uteroglobin, .beta.-actin or tyrosinase promoters. The particular promoter is not critical to the present, except in the case where the object is to obtain expression in only selective cell types. In this case, the promoter is selected to be one which is only active in the selected cell type. Examples of **tissue** specific promoters include, but are not limited to, .alpha. S1- and .beta.-casein promoters which are specific for mammary **tissue** (Platenburg et al, Trans. Res., 3:99-108 (1994); and Maga et al, Trans. Res., 3:36-42 (1994)); the phosphoenolpyruvate carboxykinase promoter which is active in **liver**, kidney, adipose, jejunum and mammary **tissue** (McGrane et al, J. Reprod. Fert., 41:17-23 (1990)); the tyrosinase promoter which is active in **lung and spleen** cells, but not testes, **brain**, heart, **liver** or kidney (Vile et al, Canc. Res., 54:6228-6234 (1994)); the involucerin promoter which is only active in differentiating keratinocytes of the squamous epithelia (Carroll et al, J. Cell Sci., 103:925-930 (1992)); and the uteroglobin promoter which is active in **lung** and endometrium (Helftenbein et al, Annal. N.Y. Acad. Sci., 622:69-79 (1991)).

Detailed Description Text (72):
Alternatively, cell specific enhancer sequences can be used to control expression, for example human neurotropic papovirus JCV enhancer regulates viral transcription in glial cells alone (Remenick et al, J. Virol., 65:5641-5646 (1991)). Yet another way to control **tissue** specific expression is to use a hormone responsive element (HRE) to specify which cell lineages a promoter will be active in, for example, the MMTV promoter requires the binding of a hormone receptor, such as progesterone receptor, to an upstream HRE before it is activated (Beato, FASEB J., 5:2044-2051 (1991); and Truss et al, J. Steroid Biochem. Mol. Biol., 41:241-248 (1992)).

Detailed Description Text (74):
In the present invention, the live invasive bacteria can deliver eukaryotic expression cassettes encoding a gene into an animal cell or animal **tissue**. The gene may be either a foreign gene or a endogenous gene. As used herein, "foreign gene" means a gene encoding a protein or fragment thereof or anti-sense RNA or catalytic

RNA, which is foreign to the recipient animal cell or **tissue**, such as a vaccine antigen, immunoregulatory agent, or therapeutic agent. An "endogenous gene" means a gene encoding a protein or part thereof or anti-sense RNA or catalytic RNA which is naturally present in the recipient animal cell or **tissue**.

Detailed Description Text (84):
In the present invention, the live invasive bacteria can also deliver eukaryotic expression cassettes encoding a therapeutic agent to animal cells or animal **tissue**. For example, the eukaryotic expression cassettes can encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof. Alternatively, the eukaryotic expression cassettes can encode synthetic genes, which encode **tumor**-specific, transplant, or autoimmune antigens or parts thereof.

Detailed Description Text (85):
Examples of **tumor** specific antigens include prostate specific antigen (Gattuso et al, Human Pathol., 26:123-126 (1995)), TAG-72 and CEA (Guadagni et al, Int. J. Biol. Markers, 9:53-60 (1994)), MAGE-1 and yrosinase (Coulie et al, J. Immunothera., 14:104-109 (1993)). Recently it has been shown in **mice** that immunization with non-malignant cells expressing a **tumor** antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant **tumor** cells displaying the same antigen (Koeppen et al, Anal. N.Y. Acad. Sci., 690:244-255 (1993)).

Detailed Description Text (87):
Examples of autoimmune antigens include IAS .beta. chain (Topham et al, Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). Vaccination of **mice** with an 18 amino acid peptide from IAS .beta. chain has been demonstrated to provide protection and treatment to **mice** with experimental autoimmune encephalomyelitis (Topham et al, supra).

Detailed Description Text (88):
Alternatively, in the present invention, live invasive bacteria can deliver eukaryotic expression cassettes encoding immunoregulatory molecules. These immunoregulatory molecules include, but are not limited to, growth factors, such as M-CSF, GM-CSF; and cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 or IFN-.gamma.. Recently, delivery of cytokines expression cassettes to **tumor tissue** has been shown to stimulate potent systemic immunity and enhanced **tumor** antigen presentation without producing a systemic cytokine toxicity (Golumbek et al, Canc. Res., 53:5841-5844 (1993); Golumbek et al, Immun. Res., 12:183-192 (1993); Pardoll, Curr. Opin. Oncol., 4:1124-1129 (1992); and Pardoll, Curr. Opin. Immunol., 4:619-623 (1992)).

Detailed Description Text (90):
In the present invention, live invasive bacteria can also deliver eukaryotic expression cassettes encoding proteins to animal **tissue** from which they can later be harvested or purified. An example is the delivery of a eukaryotic expression cassette under the control of a mammary specific viral promoter, such as derived from **mouse** mammary **tumor** virus (ATCC No. VR731), encoding .alpha..sub.1 -antitrypsin to mammary **tissue** of a goat or sheep.

Detailed Description Text (91):
Alternatively an invasive bacteria carrying a eukaryotic expression cassette can be introduced to a **tissue** site such that it would not spread from such a site. This could be accomplished by any of several methods including delivery of a very limited dose, delivery of a severely attenuated auxotrophic strain, such as an asd mutant (Curtiss et al, supra) that will be rapidly inactivated or die, or delivery of a bacterial strain that contains attenuating lesions, such as a suicide systems (Rennell et al, supra; and Reader et al, supra) under the control of a strong promoter, such as the anerobic nirB promoter (Harborne et al, supra) which will be switched on within the recipient host **tissue**. Additionally, through use of

different species and/or serotypes multiple doses of invasive bacteria, the eukaryotic expression cassette of interest can be given to an animal so as to manipulate expression levels or product type. This approach obviates the need for specially raised transgenic animals containing **tissue** specific promoters and having tight control of expression, as is currently the case (Janne et al, Int. J. Biochem., 26:859-870 (1994); Mullins et al, Hyperten., 22:630-633 (1993); and Persuy et al, Eur. J. Bichem., 205:887-893 (1992)).

Detailed Description Text (92):
As a further alternative, single or multiple eukaryotic expression cassettes encoding **tumor**-specific, transplant, and/or autoimmune antigens, can be delivered in any single or multiple combination with eukaryotic expression cassettes encoding immunoregulatory molecules or other proteins.

Detailed Description Text (94):
The invasive bacteria containing the eukaryotic expression cassette can be used to infect animal cells that are cultured in vitro. The animal cells can be further cultured in vitro, and the cells carrying the desired genetic trait can be enriched by selection for or against any selectable marker introduced to the recipient cell at the time of bactofection. Such markers may include antibiotic resistance genes, e.g., hygromycin, or neomycin, selectable cell surface markers, or any other phenotypic or genotypic element introduced or altered by bactofection. These in vitro-infected cells or the in vitro-enriched cells can then be introduced into animals intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host **tissue**.

Detailed Description Text (109):
HeLa cells (ATCC No. CCL-2) were grown on plastic **tissue** culture plates at 37.degree. C. in 5% (v/v) CO.sub.2 in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2.0 mM L-glutamine, 1.0 mM L-pyruvate, 50 U/ml penicillin and 50 .mu.g/ml streptomycin (hereinafter "RPMI/FBS"). 24 to 48 hours prior to bactofection, the HeLa cells were trypsinized with 0.25% (w/v) trypsin containing 1.0 mM EDTA, and split by limiting dilution such that they were 40-60% confluent at the time of the experiment.

Detailed Description Text (127):
Thus, it is evident that the method of the present invention is not restricted to one animal cell type, but is applicable to animal cells derived from various **tissues**.

Detailed Description Text (150):
Delivery Of A Reporter Gene in vivo to Animal **Tissue**

Detailed Description Text (151):
In order to demonstrate that bactofection can occur in vivo, restrained **mice** (Balb/c) were intranasally inoculated with 5.times.10.sup.6 viable S. flexneri .DELTA.aro.DELTA.virG containing either p.beta.-gal+SV or p.beta.-gal-SV in a volume of 10 .mu.l of PBS. 48 hours after inoculation, the **mice** were sacrificed, **lung tissue** collected and frozen to -70.degree. C. Cryosections (5.0 .mu.M) were prepared, fixed, and then stained overnight for .beta.-gal activity as described above (Hawley-Nelson et al, supra). Following staining, the sections were rinsed twice with PBS, then sealed under coverslips.

Detailed Description Text (152):
Blue-staining .beta.-gal-positive cells were visible per **lung** section infected with p.beta.-gal+SV, but not those infected with p.beta.-gal-SV.

Detailed Description Text (162):
In order to show another example of in vivo use of bactofection, 5.times.10.sup.7 S. flexneri .DELTA.aro .DELTA.virG containing the pCEP4::gp160 plasmid construct

were administered intranasally to restrained Balb/c **mice**. 14 days following bactofection, the **mice** were sacrificed and **spleens** collected.

Detailed Description Text (164):
Splenocytes **isolated from mice** bactofected with plasmid pCEP4::gp160, containing the gene for HIV-1 gp160, showed a seven-fold stimulation, while splenocytes from control (pCEP4) bactofected **mice** showed no response.

Other Reference Publication (7):
Wolff et al, "Direct Gene Transfer into **Mouse** Muscle In Vivo", Science, 247:1465-1468 (1990).

Other Reference Publication (8):
Gillies et al, "A **Tissue**-Specific Transcription Enhancer Element is Located in the Major Intron of a Rearranged Immunoglobulin Heavy Chain Gene", Cell, 33:717-728 (1983).

CLAIMS:

5. The method of claim 1, wherein said invasive bacteria is selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

7. The method of claim 1, wherein said invasive bacteria is selected from the group consisting of Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., Leishmania spp. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

23. The method of claim 15, wherein said invasive bacteria is selected from the group consisting of Shigella spp, **Listeria** spp., Rickettsia spp and enteroinvasive Escherichia coli.

24. The method of claim 15, wherein said invasive bacteria is selected from the group consisting of Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Franciesella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., Leishmania spp. and Erysipelothrix spp. which have been genetically engineered to mimic the invasion properties of Shigella spp., **Listeria** spp., Rickettsia spp., or enteroinvasive E. coli spp.

Term	Documents
LIVER	210683
LIVERS	12836
LUNG	188956

LUNGS	67332
SPLEEN	78818
SPLEENS	15399
BRAIN	223897
BRAINS	19333
TUMOR	218607
TUMORS	129040
TUMOUR	55720
(L28 and (liver or lung or spleen or brain or tumor or tissue)).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	16

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